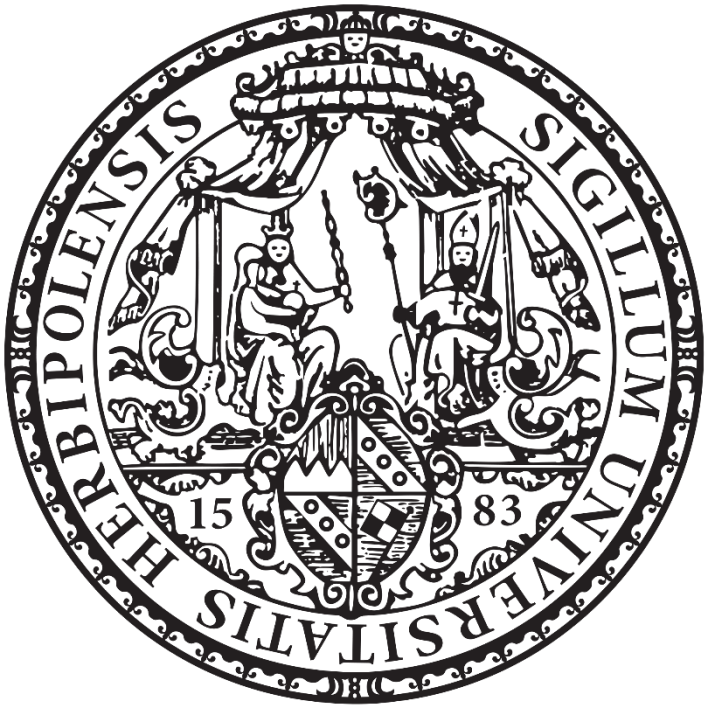
Behavioural resistance to *Varroa destructor* in the Western honeybee *Apis mellifera*

Mechanisms leading to decreased mite reproduction

Resistenzverhalten der Westlichen Honigbiene *Apis mellifera* gegen *Varroa destructor*

Zu verringerter Milbenreproduktion führende Mechanismen



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Summary

The Western Honeybee (*Apis mellifera*) is among the most versatile species in the world. Its adaptability is rooted in thousands of the differently specialized individuals acting jointly together. Thus, bees that are able to handle a certain task or condition well can back up other individuals less capable to do so on the colony level. *Vice versa*, the latter individuals might perform better in other situations. This evolutionary recipe for success ensures the survival of colonies despite challenging habitat conditions. In this context, the ectoparasitic mite *Varroa destructor* reflects the most pronounced biotic challenge to honeybees worldwide. Without proper treatment, infested colonies rapidly dwindle and ultimately die. Nevertheless, resistance behaviours against this parasite have evolved in some populations through natural selection, enabling colonies to survive untreated. In this, different behaviours appear to be adapted to the respective habitat conditions and may complement each other.

Yet, the why and how of this behavioural response to the mite remains largely unknown. My thesis focuses on the biological background of *Varroa*-resistance traits in honeybees and presents important findings for the comprehension of this complex host-parasite interaction. Based on this, I draw implications for both, applied bee breeding and scientific investigations in the field of *Varroa*-resistance. Specifically, I focus on two traits commonly found in resistant and, to a lower degree, also mite-susceptible colonies: decreased mite reproduction and the uncapping and subsequent recapping of sealed brood cells.

Examining failures in the reproductive success of mites as a primary mechanism of *Varroa*-resistance, I was able to link them to specific bee behaviours and external factors. Since mite reproduction and the brood rearing of bees are inevitably connected, I first investigated the effects of brood interruption on the reproductive success of mites. Brood interruption decreased the reproductive success of mites both immediately and in the long term. By examining the causes of reproductive failure, I could show that this was mainly due to an increased share of infertile mites. Furthermore, I proved that interruption in brood rearing significantly increased the expression of recapping behaviour. These findings consequently showed a dynamic modulation of mite reproduction and recapping, as well as a direct effect of brood interruption on both traits. To further elucidate the plasticity in the expression of both traits, I studied mite reproduction, recapping behaviour and infestation levels over the course of three years. The resulting extensive dataset unveiled a significant seasonal variation in mite reproduction and recapping. In addition, I show that recapping decreases the reproductive success of mites by increasing delayed developing female offspring and cells lacking male offspring. By establishing a novel picture-based brood investigation method, I could

furthermore show that both the removal of brood cells and recapping activity specifically target brood ages in which mite offspring would be expected. Recapping, however, did not cause infertility of mites. Considering the findings of my first study, this points towards complementary mechanisms.

This underlines the importance of increased recapping behaviour and decreased mite reproduction as resistance traits, while at the same time emphasising the challenges of reliable data acquisition. To pave the way for a practical application of these findings in breeding, we then investigated the heritability (i.e., the share of genotypic variation on the observed phenotypic variation) of the accounted traits. By elaborating comparable test protocols and compiling data from over 4,000 colonies, we could, for the first time, demonstrate that recapping of infested cells and decreased reproductive success of mites are heritable (and thus selectable) traits in managed honeybee populations.

My thesis proves the importance of recapping and decreased mite reproduction as resistance traits and therefore valuable goals for breeding efforts. In this regard, I shed light on the underlying mechanisms of both traits, and present clear evidence for their interaction and heritability.

Zusammenfassung

Die Westliche Honigbiene (*Apis mellifera*) zählt zu den anpassungsfähigsten Arten der Welt. Diese Anpassungsfähigkeit liegt in der Zusammenarbeit tausender unterschiedlich spezialisierter Individuen begründet. Auf Volksebene können Bienen, die mit einer bestimmten Aufgabe oder Situation gut umgehen können, andere Individuen, die dies weniger gut können, absichern. Andererseits können Letztere womöglich mit anderen Situationen besser umgehen. Dieses evolutionäre Erfolgskonzept sichert das Überleben der Völker selbst unter herausfordernden Habitatbedingungen. Die ektoparasitäre Milbe *Varroa destructor* stellt in diesem Zusammenhang weltweit die größte biotische Herausforderung dar. Ohne entsprechende Behandlung siechen die Völker rasch dahin und sterben schlussendlich. In einigen Populationen haben sich jedoch Resistenzmechanismen durch natürliche Selektion herausgebildet, die es den Völkern ermöglichen, ohne Behandlung zu überleben. Die verschiedenen Verhaltensweisen scheinen dabei an die jeweiligen Habitatbedingungen angepasst zu sein und sich gegenseitig zu ergänzen.
Was diese Reaktion auf die Milben auslöst und wie sie funktioniert ist allerdings noch weitestgehend unbekannt.

Meine Dissertation fokussiert den biologischen Hintergrund von *Varroa*-resistenzmechanismen bei Honigbienen und stellt dabei wichtige Erkenntnisse zum Verständnis dieser komplexen Parasit-Wirt-Beziehung vor. Darauf aufbauend leite ich Implikationen für die angewandte Bienenzucht und wissenschaftliche Untersuchungen auf dem Gebiet der *Varroa*-resistenz ab.

Hierbei konzentriere ich mich insbesondere auf zwei Merkmale, die häufig in resistenten Völkern zu finden sind: die reduzierte Milbenreproduktion und das Entdeckeln und Wiederverdeckeln bereits verschlossener Brutzellen. Beide Merkmale treten in geringerem Umfang auch in milbenanfälligen Populationen auf und sind daher von besonderem Interesse für jedwede Zuchtbemühung mit dem Ziel der *Varroa*-resistenz.

Durch die Untersuchung von Fehlern in der Reproduktion der Milben, konnte ich diesen Hauptmechanismus der *Varroa*-resistenz mit Verhaltensweisen der Bienen, sowie äußeren Faktoren in Verbindung setzen. Da die Milbenvermehrung untrennbar mit der Brutaufzucht der Bienen verbunden ist, habe ich zunächst die Einflüsse von Brutunterbrechungen auf den Vermehrungserfolg der Milben untersucht. Diese Untersuchung zeigte auf, dass Brutunterbrechungen den Vermehrungserfolg der Milben sowohl kurzfristig, als auch langfristig herabsetzen. Durch die Untersuchung der jeweils zugrundeliegenden Ursachen

gescheiterter Milbenreproduktion konnte ich zeigen, dass dies vor Allem auf einen gesteigerten Anteil infertiler Milben zurückzuführen war. Des Weiteren konnte ich beweisen, dass die Unterbrechung der Brutaufzucht die Ausprägung des Wiederverdeckelns signifikant verstärkte. Folglich zeigten diese Ergebnisse eine dynamische Anpassung der Milbenreproduktion und des Wiederverdeckelns, sowie einen direkten Einfluss der Brutunterbrechungen auf beide Eigenschaften. Um die Plastizität der Ausprägung beider Merkmale genauer zu erklären, untersuchte ich daraufhin drei Jahre lang die Milbenvermehrung, das Verhalten des Wiederverdeckelns, sowie die Befallsentwicklung. Daraus resultierte ein umfangreicher Datensatz, der eine signifikante saisonale Variation der Milbenvermehrung und des Wiederverdeckelns belegte. Ich konnte außerdem eindeutig beweisen, dass das Wiederverdeckeln den Reproduktionserfolg der Milben herabsetzt, indem es die Anteile von verzögert heranwachsenden weiblichen Nachkommen und fehlenden Männchen steigert. Durch Anwendung einer neuartigen Bild-basierten Methode der Brutuntersuchung, konnte ich darüber hinaus zeigen, dass sich sowohl das Ausräumen, als auch das Wiederverdeckeln von Brutzellen auf Brutalter konzentriert, in denen Milbennachwuchs erwartet werden würde. Das Wiederverdeckeln trug jedoch nicht zur Infertilität der Milben bei, was zusammen mit den Ergebnissen meiner ersten Untersuchung auf komplementäre Mechanismen hinweist. Dies unterstreicht die Bedeutung des Wiederverdeckelns und der verminderten Milbenreproduktion als Resistenzmechanismen, hebt aber gleichzeitig auch die Herausforderungen einer verlässlichen Datenerhebung hervor. Um den Weg für die praktische Anwendung dieser Erkenntnisse in der Zuchtarbeit zu ebnen, untersuchten wir daraufhin die Erblichkeit (den Anteil der genotypischen Variation an der beobachteten phänotypischen Variation) der betrachteten Merkmale. Durch das Erarbeiten vergleichbarer Prüfprotokolle und Zusammenführen von Daten aus über 4000 Völkern, konnten wir erstmalig zeigen, dass das Wiederverdeckeln befallener Zellen und der verminderte Vermehrungserfolg der Milben erbliche und damit selektierbare Merkmale in bewirtschafteten Honigbienenpopulationen sind.

Meine Dissertation beweist die Relevanz des Wiederverdeckelns und der verminderten Milbenreproduktion als Resistenzmerkmale und damit lohnende Ziele für Zuchtbemühungen. In diesem Zusammenhang beleuchtete ich verschiedene Mechanismen, die der Ausprägung beider Merkmale zugrunde liegen und lieferte eindeutige Beweise für deren Interaktion und Erblichkeit.

Contents

A list of abbreviations is given in the Appendix and Table 1 (Subchapter 1.2.1).

They alone raise children in common,
own the roofs of their city as one,
and pass their life under the majesty of the law.
They alone know fatherland and settled home,
and in summer, remembering the winter to come,
spend toilsome days, storing their gains for all.

Virgil, Georgics Book IV

Chapter I

Introduction

Honeybees (*Apis spp.*) have mastered the art of collaboration. The social structure of colonies shaped by division of labour has enabled their adaptation to various biotic and abiotic factors (Seeley 2014; Ruttner 1988; Winston 1987), to the astonishment and admiration of human societies since the beginnings of apiculture. Based on the diversity of different worker abilities in the colony, such adaptations typically evolve over long periods in nature (Ruttner 1988). However, rapid changes in the habitat, often as a result of anthropogenic activity, can pose a challenge of fast adaptation as the only alternative to extinction. Recently, the most prominent example of adaptation to such rapid change has been the emergence of resistance behaviours of Western honeybees (*Apis mellifera* L.) against the fast-spreading mite *Varroa destructor* ANDERSON & TRUEMAN, hereafter referred to as "*Varroa*" (Mondet et al. 2020a; Oddie et al. 2018). Such behavioural patterns are displayed by a few individuals on colony level, which is however sufficient to ensure the survival of the whole colony by keeping infestation levels under a lethal threshold (Mondet et al. 2020a).

Varroa, an ectoparasitic mite, has passed over from the Eastern honeybee (*Apis cerana* FABRICIUS), its initial host native to Asia, to the nearly ubiquitous Western honeybee, hereafter broadly referred to as "honeybees" (Rosenkranz et al. 2010). From the first half of the twentieth century onwards, this host shift made possible the rapid dispersion of *Varroa* around the globe (Le Conte et al. 2020; Wilfert et al. 2016; Rosenkranz et al. 2010), challenging locally adapted honeybee populations in different climatic zones (Locke 2016; Wilfert et al. 2016). The parasite weakens the honeybee host dramatically by direct feeding and virus vectoring (Ramsey et al.

2019; Rosenkranz et al. 2010), thereby frequently causing ultimate colony losses in case of high infestation levels (Genersch et al. 2010). Since the arrival of this new pest, most honeybee populations have therefore been treated by beekeepers through regular acaricide applications to prevent such losses (Traynor et al. 2020; Büchler et al. 2010). From these treatments, in turn, a range of new problems has arisen, including acaricide residues in hive products (Wallner 1999) and acaricide resistance in *Varroa* (Milani 1999), which has rendered several active compounds ineffective and unusable. The most significant side effect of this management practice, however, has been the inhibition of natural selection towards honeybees able to cope with mite infestation on their own (Traynor et al. 2020; Neumann & Blacquière 2017; Büchler et al. 2010). On the other hand, the basic mechanisms of evolution could work unhindered in a few untreated (i.e., mostly less managed) populations, resulting in a natural selection of resistance mechanisms enabling them to survive without treatment (Mondet et al. 2020a; Locke 2016). Thus, naturally selected honeybee populations today act as a benchmark for targeted selection in bee breeding, which aims for an increased resistance to *Varroa* in managed honeybee stocks already selected for other traits desirable in beekeeping (e.g., gentleness and honey production [Büchler et al. 2010; Rinderer et al. 2010]).

All resistance mechanisms are intrinsically linked to the life cycle of *Varroa*, which will be described in the following sections.

1.1 The life cycle of *Varroa destructor*

The life cycle of *Varroa* comprises two distinct phases: 1) the reproductive phase inside worker and drone brood cells of the host and 2) the dispersal phase on adult honeybees (Fig. 1, [Traynor et al. 2020; Nazzi & Le Conte 2016; Rosenkranz et al. 2010]).

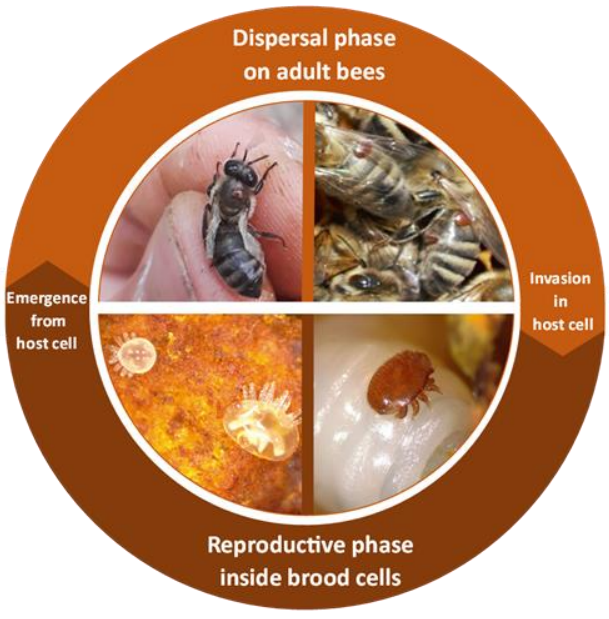

Figure 1: Schematic illustration of the dichotomous life cycle of *Varroa destructor*. Upper half: The dispersal phase on adult honey bees lasts for approximately 7 days after emerging from the host cell. Pictures display adult bees parasitised by adult *Varroa* mites. Lower half: The reproductive phase starts after the invasion in suitable brood cells and lasts for approximately 12 or 15 days in worker or drone brood, respectively. Pictures display a mother mite inside freshly capped worker brood (lower right) and mite offspring 9 days after capping (lower left). Modified after Uzunov et al. (2023).

The duration of the reproductive phase is predefined by the development of the host cells. It therefore lasts approximately 12 and 15 days after cell capping in worker and drone cells, respectively. To begin the process, mites normally invade cells up to two days prior to capping. It is not exactly known how often mother mites invade cells for separate reproductive cycles during their life time, i.e., how many consecutive reproductive phases they may attempt. Under laboratory conditions, up to seven reproductive phases have been described (De Ruijter 1987), while an average of two to three cycles appears to be realistic under field conditions (Martin & Kemp 1997; Fries & Rosenkranz 1996).

During the subsequent dispersal phase, mites likewise rely on their host, typically hiding between the abdominal sternites of workers (Nazzi & Le Conte 2016). The duration of this phase thereby depends on manifold factors such as colony strength and brood presence (Nazzi & Le Conte 2016). If brood stages suitable for invasion are present, most mites undergo a dispersal phase of up to seven days (Sammataro et al. 2000; Boot et al. 1993). However, in the absence of brood, e.g., during natural brood breaks in wintertime, this may expand to several months.

Both phases of the mites' life cycle are described in detail below.

1.1.1 Reproductive phase

The reproductive phase of *Varroa* is highly dependent on the brood development of honeybees (Nazzi & Le Conte 2016; Rosenkranz et al. 2010). It begins approximately two days before brood capping, when *Varroa* mites invade brood cells in 5th instar larval stadium (Nazzi & Le Conte 2016; Rosenkranz et al. 2010). In this, drone brood is strongly preferred compared to worker brood due to a longer post-capping duration and stronger olfactory cues (Boot et al. 1995b; Fuchs 1990). After capping, the reproductive phase lasts approximately 12 or 15 days in worker and drone cells, respectively. Olfactory cues from the larval food (Nazzi et al. 2004) as well as from the host larvae (Le Conte et al. 1989) appear to be the main attractants leading mites into the brood cells. Here, they first dwell in the larval food, breathing through their peritreme, a snorkel-like respiratory organ (Rosenkranz et al. 2010). This first arrestment step after invasion is commonly interpreted as a form of hiding from adult bees displaying resistance traits (Rosenkranz et al. 2010) and followed by a distinct series of behaviours elicited by different host factors (Nazzi & Le Conte 2016). Having consumed the brood food, the host larva begins to spin a cocoon for pupation (Donzé & Guerin 1994). The mite dodges the moving praepupa to avoid getting trapped in the cocoon, a behaviour which, again, appears to be fostered by olfactory cues from the cocoon itself (Donzé et al. 1998). After pupation of the host, the mite creates the fecal accumulation site by defecating repeatedly on the cell wall, usually

near the eighth segment of the host pupa (Donzé & Guerin 1994). From now on, this spot serves as a constant orientation point for the mother mite as well as a meeting point for the offspring produced later (Donzé & Guerin 1994). Near this spot, the mother mite creates a communal feeding site both for herself and for her subsequent offspring by piercing a hole in the bee cuticula (Donzé & Guerin 1994). Since the chelicerae of mite offspring would not be suitable for such a puncture, this behaviour of the mother mite reflects a form of parental care necessary for the survival of her descendants (Rosenkranz et al. 2010). After feeding, mites return to the fecal accumulation site as their main spot of residence within the cell (Donzé & Guerin 1994). This feeding activity reflects the major damage caused by *Varroa*, both directly through haemolymph and fat body consumption (Ramsey et al. 2019) as well as indirectly by pathogen transmission (Rosenkranz et al. 2010). Consecutively, the mother mite lays the first egg (Fig. 2), which usually develops into a male (Martin 1994). This first oviposition takes place between 60 and 70 hours after cell capping in both worker and drone brood (Donzé & Guerin 1994; Martin 1994). In worker brood, it is followed by up to four eggs subsequently laid in 30-hour intervals which develop into female mite offspring (Rosenkranz et al. 2010; Martin 1994). In drone brood, an additional female egg can be laid due to the longer development time of the host cell (Rosenkranz et al. 2010; Martin 1995). In this, the haplo-diploid sex determination system of *Varroa* causes unfertilised eggs to develop into males, while fertilised eggs develop into females (Rehm & Ritter 1989). Notably, oviposition is highly dependent on kairomones originating from the host larvae (Nazzi & Le Conte 2016). Although not fully identified, these triggers inducing the oviposition are present up to 12 hours and 36 hours after cell capping in worker and drone brood, respectively (Frey et al. 2013; Rosenkranz & Garrido 2004). Mites entering the cell after this period remain infertile, i.e., they lay no eggs at all (Frey et al. 2013). Again, this inhibition of oviposition appears to be caused by yet unknown kairomones of the host pupa (Rosenkranz & Garrido 2004).

After oviposition, the ontogenesis of mite offspring takes 154 h in males and around 134 h in females to reach the adult molt stage, including a proto- and a deutonymph stage in both sexes (Martin 1994). The reproduction schedule of the mite is thus tightly bound to the capped period of host brood development (Büchler & Drescher 1990), since both the maturation and the mating of any offspring must take place before the parasitised bee hatches (Ziegelmann et al. 2013; Rosenkranz et al. 2010). Female and male mites reach sexual maturity with their final molt (Ziegelmann et al. 2013). Due to the earlier deposition of the unfertilised egg, male mites reach maturity first, although they display a longer development time (Donzé & Guerin 1994). They therefore wait for around 20 hours at the fecal accumulation site for the mature female offspring and immediately attempt to mate with them once they arrive (Donzé et al. 1996;

Ziegelmann et al. 2013). In the default case of one reproducing mother mite per cell, daughter mites thereby mate with their brother, given the offspring constellation of one male and several females. Mating occurs repeatedly within the first 24 hours after daughter mites have reached maturity (Ziegelmann et al. 2013) and is followed by a spermatozoa capacitation period of around 5 days (Häußermann et al. 2016). However, the spermatozoa capacitation partly takes place after mated daughter mites have left the cell alongside with their mother and the hatching host bee (Häußermann et al. 2016). Nevertheless, the time frame given by the development of the host is a crucial limitation for the number of mated daughters raised per mother, i.e., her reproductive success. It limits *Varroa*-reproduction to an average rate of 1.3–1.45 and 2.2 –2.6 mated daughters per brood cycle in single-infested worker and drone brood, respectively (Martin 1994, 1995). However, the number of mated daughters per mother decreases with an increasing number of mother mites in multiple-infested worker and drone cells, which can occur frequently in highly infested colonies (Fuchs & Langenbach 1989). Male mites as well as immature daughters are unable to survive outside the host cell and therefore die after the parasitised bee has hatched (Rosenkranz et al. 2010). Mother mites and mated daughters (Nazzi & Le Conte 2016), as well as virgin mature daughters (Häußermann et al. 2020), pass on to the following dispersal phase (Fig. 2).

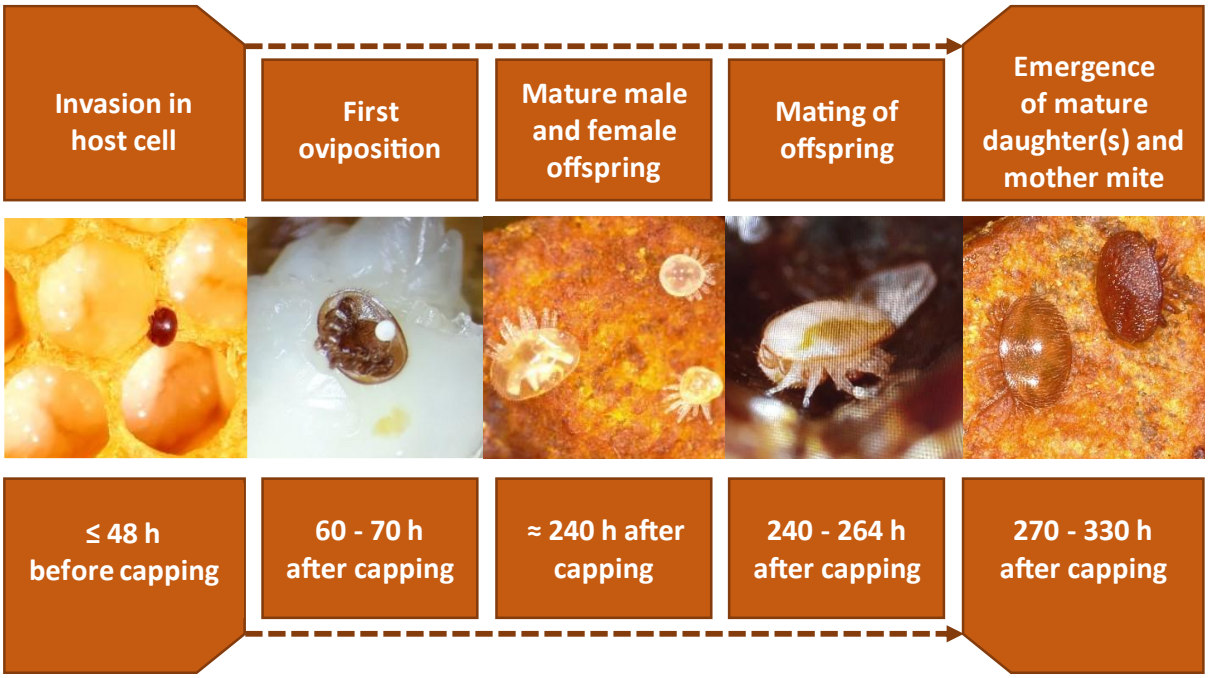

Figure 2: Schematic schedule of the reproductive phase of *Varroa destructor*. The invasion of mother mites into brood cells in the 5th instar larval stage (ca. 48 h prior capping) is followed by the first oviposition approximately 70 h post capping. Subsequent eggs are laid in 30 h intervals. Around 240 h after capping the male and the first daughter mite reach maturity and attempt to mate within the next 24 h. Approximately 270 or 330 h post capping in worker and drone cells, respectively, mother mites emerge from the host cell with the mature daughter mites and enter the following dispersal phase. Durations according to Ziegelmann et al. (2013) and Donzé & Guerin (1994). See 1.1.1 Reproductive phase for detailed description.

1.1.2 Dispersal phase

Having emerged with the host bee from its brood cell, *Varroa* starts the dispersal phase by moving onto another bee (Fig. 1). The mites usually hide between the abdominal sternites, again feeding on their host by piercing through a metasomatic intersegmental membrane (Ramsey et al. 2019; Nazzi & Le Conte 2016). In doing so, mites prefer nurse bees over foragers or freshly hatched bees, although this can vary with the level of infestation on a colony scale (Cervo et al. 2014; Kuenen & Calderone 1997). The host finding behaviour again relies on olfactory cues deriving from the cuticula profile of the bees (Nazzi & Le Conte 2016; Cervo et al. 2014). This preference for nurse bees increases mites' chances to reach a brood cell suitable for invasion within the following days. At the same time, it decreases the risk of facing out-hive dangers, e.g., getting carried away by a forager which dies in the field (Nazzi & Le Conte 2016). In addition, mites have been observed to reproduce more successfully after parasitising nurse bees as adult hosts, which points to further benefits of this worker class as food source during the dispersal phase (Xie et al. 2016; Stürmer & Rosenkranz 1994). On the other hand, a horizontal transmission of mites between individual colonies frequently occurs through drifting or robbing foragers (Frey & Rosenkranz 2014), which proves the transfer of mites by this worker class. In this way, *Varroa* opens up new hosts by "hitchhiking" on foragers to other colonies, which in fact might be crucial for survival if the natal colony is about to collapse due to a high infestation level. Correspondingly, this phenomenon occurs mainly under high infestation rates at the colony level, when the preference for nurse bees becomes less pronounced and mites increasingly move onto forager bees (Cervo et al. 2014). This is, however, rather due to a change in the chemical cuticular signature of worker bees than to a change in mite behaviour itself (Cervo et al. 2014). Under a high *Varroa*-burden, the chemical profiles of forager and nurse bees tend to overlap, which leads mites in their dispersal phase more often to choose foragers as hosts (Cervo et al. 2014).

Apart from the potential of such transfers to new hosts, not much is known about the relevance of the dispersal phase for the following reproductive attempts of *Varroa*. Under artificial conditions, up to seven reproductive phases gaining offspring have been reported without any dispersal phase in between (De Ruijter 1987). Under natural conditions, however, dispersal phases seem to last at least several days (Sammataro et al. 2000; Boot et al. 1993), while no effect of the respective duration was reported on the success of following reproductive phases (Piou et al. 2016; Boot et al. 1995a). In freshly mated daughter mites, at least, the first dispersal phase might be crucial to accomplish spermatozoa capacitation before invading a brood cell for the subsequent reproductive phase (Häußermann et al. 2016).

1.2 Resistance or tolerance?

Honeybees' approaches to cope with *Varroa destructor*

The life cycle of mites in general and their complex reproduction biology in particular offer manifold possibilities for the honeybee host to lower the damage caused by *Varroa*. Most insights into such mechanisms have been derived from untreated yet surviving populations (Mondet et al. 2020a; Traynor et al. 2020; Oddie et al. 2018). These findings suggests that individual traits, as well as the respective set of co-occurring mechanisms, are "evolutionary-tailored" to the needs of colonies under their respective environmental conditions (Locke 2016). Therefore, various traits evolved in this way have been observed in surviving populations, which makes them role models for targeted selection (Le Conte et al. 2020; Mondet et al. 2020a; Oddie et al. 2018). These traits can broadly be divided into the fields of 1) host tolerance, describing the ability of honeybees to reduce or tolerate the damage caused by the mites, and 2) host resistance, describing the ability of individual honeybees or colonies to lower the reproductive success of mites in a way that keeps infestation under a fatal threshold (Mondet et al. 2020a). Some few studies in surviving populations have revealed true tolerance, mostly towards *Varroa*-associated viruses (Thaduri et al. 2019; Mordecai et al. 2016). Still, the great majority of surviving populations rely on resistance mechanisms, lowering the reproductive success of mites (Mondet et al. 2020a).

Thus, *Varroa*-resistance rather than *Varroa*-tolerance is the more accurate description for the main mechanisms enabling the honeybee populations in question to survive untreated. The following paragraphs therefore focus on such mechanisms and discuss the most prominent *Varroa*-resistance traits of honeybees.

1.2.1 Mite non-reproduction

The occurrence of reproductive failure in mites, i.e., of any form of unsuccessful reproductive attempt, constitutes the most important driver for reduced mite population growth and correspondingly low infestation levels in *Varroa*-resistant honeybees (Martin et al. 2020; Mondet et al. 2020a; Harbo & Hoopingarner 1997). This absence of successful reproduction was initially termed suppressed mite reproduction (SMR [Harbo & Harris 1999b]), but nowadays is mostly referred to as mite non-reproduction (MNR), following the suggestions of Mondet et al. (2020a). However, various different yet sometimes overlapping names and definitions have been used in the context of reproductive failure of *Varroa*. This variety of terms might easily lead to confusion about the addressed definition, since the same term is used for different reproductive conditions and *vice versa* (displayed in Tab 1). In general, I follow the most commonly used definition of Mondet et al. (2020a), MNR, which

is congruent with the formerly used and widely known term SMR in a broader sense (SMR s.l.). In this sense, MNR describes a single infested cell hosting a mother mite with either 1) no offspring (infertile), 2) only female offspring (no male) or 3) progeny which is too young to reach maturity (delayed) before the host cell is expected to hatch (see Chapter 2, Fig. 3). Thus, MNR reflects a binomial factor (reproduction either successful or not) and does not account for possible differences in the fecundity of mites (i.e., the number of viable offspring raised per mite, see 1.1.1 Reproductive phase). Nevertheless, it currently is the most commonly accounted resistance trait (Le Conte et al. 2020) and was found to be a key feature of several populations surviving untreated (Luis et al. 2022; Grindrod & Martin 2021; Mondet et al. 2020a). The occurrence of different forms of MNR (infertile, no male or delayed), have been less intensively studied than MNR *per se* (Mondet et al. 2020b). Yet, their contribution to the overall MNR-values differ considerably between populations (Scaramella et al. 2023; Mondet et al. 2020b) and thus likely reflect different background mechanisms leading to reproductive failure in *Varroa*.

Increased levels of mite infertility have been attributed to host brood factors (Scaramella et al. 2023), as well as to behaviours of adult bees (Harbo & Harris 2005). Since the oogenesis of *Varroa* is highly dependent on the right host signals (Frey et al. 2013), infertility seems to be mainly linked to mismatches between kairomones emitted by the brood and invading mites (Scaramella et al. 2023; Sprau et al. 2021; Frey et al. 2013; Nazzi & Le Conte 2016; Nazzi & Milani 1996). This could either be induced by adaptive changes of the kairomone profile of the brood itself (Scaramella et al. 2023), or result from mismatches between brood age and mite invasion (Frey et al. 2013). While the first case would solely be induced by brood factors, the latter one might partly result from adult bee behaviours (e.g., recapping) leading to mite invasion in unsuitable brood ages (Oddie et al. 2018). Mature daughter mites which failed to mate in their host cell have also been discussed as a source of infertile mites in following reproductive attempts (Martin et al. 1997). However, Häußermann et al. (2020) pointed out later that most of these virgins lay at least one male egg. As reviewed by Rosenkranz et al. (2010), susceptible honeybee populations commonly show infertility levels of up to 20 % within their mite population. An increase of this proportion, as frequently found in resistant populations (Rosenkranz et al. 2010), could also result from adult bees selectively removing fertile mites from their brood cells (*Varroa*-sensitive hygiene; [Mondet et al. 2020a; Oddie et al. 2018]). Likewise, other targeted behaviours towards reproducing mites (e.g., recapping [Oddie et al. 2018]) could lead to a removal of the first two descendants (son and first daughter), whose larval stages are typically located near the cell cap (Donzé & Guerin 1994). This would also result in MNR, caused either by the lack of male offspring (removal of the male) or delayed

reproduction (removal of the eldest daughter). In addition, Locke et al. (2012) assumed that the delay in mite reproduction could derive from suppressed oviposition, which was shown to be induced by volatiles emitted by the host brood (Scaramella et al. 2023; Frey et al. 2013; Milani et al. 2004). Delayed reproduction is overall the most commonly found form of MNR in European populations, while the absence of males occurs comparatively rarely (Scaramella et al. 2023; Mondet et al. 2020b). However, the lack of vital male offspring was found to account for nearly 80 % of MNR in Ethiopian honeybees (Gebremedhn et al. 2019), which again points to different mechanisms causing MNR. Beside the removal by adult bees, such a deficiency of adult males could be caused by mismatched brood triggers leading mother mites to lay only female eggs (Rosenkranz & Garrido 2004), or the inability of immature males to reach the feeding site (Martin & Kryger 2002; Donzé & Guerin 1994).

In addition to host adaptations that lower the reproductive success of *Varroa* in one way or the other, MNR is most likely affected by a set of environmental factors (reviewed in [Rosenkranz et al. 2010]). Although the trait was described to be heritable in a small crossing study (Harbo & Harris 1999b), the repeatability of MNR was thus found to be modest at best in more recent investigations (Büchler et al. 2020; Eynard et al. 2020). It thus remains important to note that MNR measurements reflect a composition of several effects rather than a single behavioural trait.

Table 1: Overview of commonly used (and partly overlapping) definitions of resistance traits related to mite reproduction and brood cell removal.
Definitions for MNR and SMR used in this thesis are indicated in bold.

Term for resistance trait	Definition according to the respective authors	Source
SMR (s.l.) – suppressed mite reproduction	Failure of reproduction characterised by either 1) no offspring of living (infertile) or dead mother mite, 2) only male offspring, or 3) delayed reproduction resulting in progeny hatching too late to mature.	Harbo & Harris 1999a
	In addition to the characteristics defined by Harbo and Harris (1999a), later publications also assigned the absence of male offspring to SMR (s.l.).	**Büchler et al. 2020; Büchler et al. 2017**
MNR – mite non-reproduction	**Any form of failed reproduction characterised by the lack of at least one mated adult daughter at the end of the reproductive cycle, i.e., when the mother mite leaves the host cell. Therefore, it matches the description of SMR (s.l.) given by Harbo and Harris (1999a). However, SMR (s.s.) was described as a distinctive form of MNR (see below) by Mondet et al. (2020a).**	**Mondet et al. 2020a**
SMR (s.s.) – suppressed mite reproduction	Redefined as a special form of MNR which is solely related to brood factors of the host and does not include behaviours performed by adult bees	Mondet et al. 2020a

Continued on the next page

Term for resistance trait	Definition according to the respective authors	Source
DMR (a/b) – decreased mite reproduction	Generic term for either of the following terms RMR or MNR which might be specified with "a" or "b" if induced by traits of adult bees or brood, respectively	Von Virag et al. 2022
RMR – reduced mite reproduction (synonym: fecundity-based DMR)	Decreased mite fecundity, i.e., fewer mature daughters raised per mother mite	
MNR – mite non-reproduction (synonym: infertility based DMR)	Completely failed reproduction through lack of mature daughters.	
VSH – *Varroa*-sensitive hygiene	Behaviour of adult bees, which lethally remove infested pupae from capped brood.	Harris 2007; Harbo & Harris 2005
REC – recapping	Behaviour of adult bees, which open and reseal capped brood cells without harming the pupae inside.	Oddie et al. 2018
RECall – recapping of all brood cells	Proportion of REC based on all investigated brood cells (i.e., infested and uninfested cells)	Guichard et al. 2022
RECinf – recapping of infested brood cells (synonym: targeted recapping)	Proportion of REC based only on infested brood cells (i.e., targeted REC).	Oddie et al. 2021

1.2.2 *Varroa*-sensitive hygiene

The selective removal of *Varroa*-infested brood by adult worker bees (Fig. 3) reflects a specific form of brood hygiene behaviour and consequently has been termed *Varroa*-sensitive hygiene (VSH, [Mondet et al. 2020a; Harbo & Harris 2005]). Such behaviour has long been known from *Apis cerana*, where it restricts *Varroa*-reproduction to drone brood cells. This greatly decreases the infestation growth and harm caused by the mite (Grindrod & Martin 2023). In *Apis mellifera*, VSH was later identified as a main driver of reproductive failure of mites (Harbo & Harris 2005), after earlier studies had focussed on the occurrence of failed reproduction *per se* (Harbo

& Harris 1999). Step by step, deeper understanding of causes (behaviours like VSH) and effects (MNR, [Harris 2007; Harbo & Harris 2005]) has led to changing definitions of resistance mechanisms over time (reviewed by Mondet et al. [2020a]). Thus, the terms VSH and MNR are sometimes mistakenly thought to be interchangeable, despite the fact that they refer, respectively, to a resistance behaviour of adult bees leading to reduced reproductive success of mites or the outcome of different such effects causing reproductive failure (Tab. 1).

VSH is one of the best-studied resistance traits of honeybees, yet large parts of the behavioural cascade involved have remained unclear (Mondet et al. 2020a; Traynor et al. 2020). The VSH activity of bees seems to focus on brood stages up to five days post capping (Harris 2007). However, the initial cause for this lethal removal of brood cells still needs to be identified. Several olfactory cues deriving from the parasitised brood are assumed to act as triggers inducing the behaviour (Traynor et al. 2020). Yet, the suspected substances are relatively involatile and therefore appear unlikely to be sensed by worker bees outside the cell cap (Traynor et al. 2020). The detection of infested brood could thus be fostered by opening the cell lid, a behavioural step that resembles the recapping trait (REC) described below (Martin et al. 2020).

In this context, the role of fertile mites as a trigger and primary target of VSH has also been discussed (Ibrahim & Spivak 2006; Harbo & Harris 2005). This behaviour, however, appears to target both fertile and infertile mites (Sprau et al. 2021; Harris et al. 2010). Nevertheless, bees apparently discriminate between living and dead mites, as they tend to remove the latter less frequently (Sprau et al. 2023). Thus, Sprau et al. (2023) assumed that several cues like odours deriving from the mite or the feeding wound, as well as distress signals from the brood might jointly contribute to the expression of VSH.

Although the underlying mechanisms are not completely understood, VSH plays an undisputed role in the survival of several untreated honeybee populations (Luis et al. 2022; Grindrod & Martin 2021; Mondet et al. 2020a), and is thus commonly seen as one of the most important resistance behaviours (Le Conte et al. 2020; Mondet et al. 2020a; Traynor et al. 2020). Rather than being a prerequisite for long-term colony survival, however, VSH appears to be one possible way to it (Locke 2016). Two separated Dutch populations of resistant bees nicely display this heterogeneity of resistance traits: Although the populations were located relatively close to each other, Panziera et al. (2017) found a sharply increased VSH expression in one of them, while the behaviour was significantly less displayed in the other when compared to susceptible control colonies, respectively.

Nevertheless, given the apparent negative effects on mite reproduction (Harbo & Harris 2005),

VSH is currently the subject of several breeding programs (Le Conte et al. 2020). Yet, their respective selection decisions are almost always based on MNR values (Mondet et al. 2020a), since the measurement of VSH is comparatively laborious. Several methods were described to account for this behaviour (Sprau et al. 2021; Bienefeld et al. 2015; Villa et al. 2009; Boecking & Drescher 1991). However, all of them rely on the inspection of brood cells and sometimes tedious artificial infestations (Sprau et al. 2021; Boecking & Drescher 1991). Thus, although the trait was found to be heritable (Sprau et al. 2023; Boecking et al. 2000), targeted breeding efforts are still greatly challenged by a lack of reliable yet labor-efficient measurements of VSH expression.

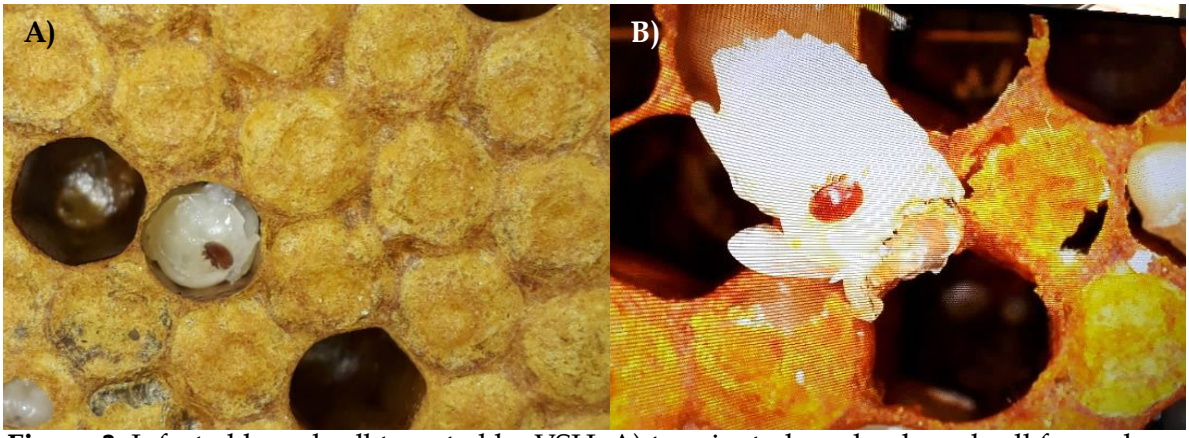

Figure 3: Infested brood cell targeted by VSH: A) terminated worker brood cell from above and B) lateral view on the half-chewed pupae. The mother mite is still present while the anterior part of the pupa (head and parts of the thorax) was already removed by worker bees.

1.2.3 Recapping of brood cells

Recapping (REC) describes a two-stage sequence of worker bee behaviours including 1) the partial opening of the wax cap and pupal cocoon of brood cells without harming the pupa developing inside (Fig. 4), followed by 2) the resealing of this hole in the cell cap with wax (Grindrod & Martin 2021a; Martin et al. 2020; Oddie et al. 2018). Increased levels of recapping on the colony level have been found in several resistant populations around the globe (Luis et al. 2022; Grindrod & Martin 2021a; Mondet et al. 2020a; Traynor et al. 2020; Oddie et al. 2018). In line with this, Oddie et al. (2021) as well as Hawkins and Martin (2021) have reported lower reproductive success of *Varroa* (MNR) in colonies which targeted infested cells with REC. Recapping appears to constitute a basal brood hygiene behaviour which is at low levels even displayed in *Varroa*-naïve colonies (Martin et al. 2020) and sharply increases after infestation of the colony (Hawkins & Martin 2021). REC generally occurs in both uninfested and infested cells, however the main activity seems to cluster around infested cells (Grindrod & Martin 2021b). In addition, the size of the opening, i.e., the effort spend by worker bees, was found to

be bigger in infested cells than in uninfested cells (Grindrod & Martin 2021b). The increased REC values found in surviving compared to susceptible and naïve populations, as well as the targeted activity towards infested cells thus point to a behavioural adaptation of honeybees to their parasite. However, the investigation of direct effects of REC on *Varroa*-reproduction has led to controversial results (Von Virag et al. 2022; Hawkins & Martin 2021; Oddie et al. 2018). Thus, it was proposed that such effects sometimes might be overshadowed by other factors, or might not be the primary cause for failures in mite reproduction (Hawkins & Martin 2021). In this context, a connection to the prominent resistance behaviour VSH (*Varroa*-sensitive hygiene) has been especially discussed, since this also involves the opening of sealed brood cells (Hawkins & Martin 2021; Mondet et al. 2020a; Oddie et al. 2018; Harris et al. 2010). The occurrence of REC therefore might be nothing more than an interruption of the behavioural cascade leading to VSH, and could therefore reasonably be seen as a proxy for this behaviour at best (Martin et al. 2020). On the other hand, for honeybees REC would be more cost-effective than VSH, since the targeted brood cells are not harmed by the behaviour, while pupae are killed in case of VSH (Le Conte et al. 2020; Oddie et al. 2018). Thus, the question whether REC reflects a proxy for VSH rather than separate resistance trait, is still being debated, keeping it in the focus of current research (Dall'Olio et al. 2022). In this context, tedious brood investigations are required to measure the occurrence of REC. To that end, brood cells are opened with fine forceps to inspect the underside of the cell cap (Büchler et al. 2017). In the case of REC, the silken pupal cocoon attached to the inner side of the cell cap has been irreversibly opened by worker bees and therefore shows distinct holes (Fig. 4, [Martin et al. 2020; Mondet et al. 2020a]).

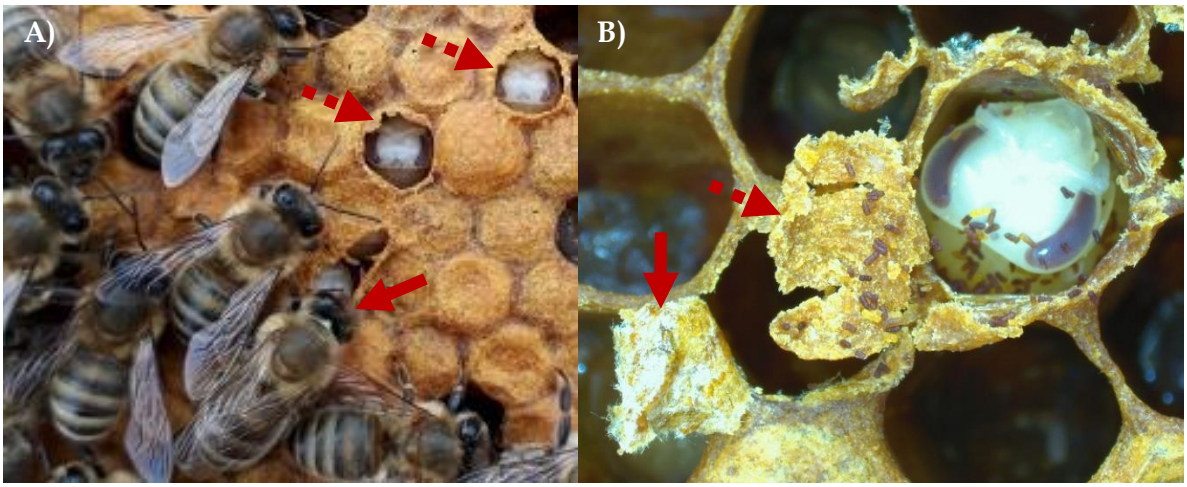

Figure 4: Recapping in worker brood cells. A) Uncapping of a brood cell by a worker bee (solid arrow) and already uncapped (bald) brood cells (dashed arrows). B) Two neighbouring cells opened during brood investigation. The left cell shows the shiny pupal cocoon on the underside of the cell lid (solid arrow) and thus was not recapped, while the cocoon is missing on the recapped lid of the right cell (dashed arrow). Note the wax moth (*Galleria mellonella* or *Achroia grisella*) faeces inside the recapped cell, which most probably triggered recapping.

Since worker bees reseal the wax cap of the cell from above, these holes in the cocoon are not visible from the outside. In the absence of REC, however, i.e., if the cell had never been opened after pupation of the larva, the cocoon has stayed intact and thus completely covers the inner surface of the cell cap (Fig. 4, [Martin et al. 2020; Mondet et al. 2020a]). Aside from data acquisition, the interpretation of phenotypic REC values remains an additional challenge. The repeatability of REC has been found to be low to moderate, depending of the population studied (Guichard et al. 2022; Büchler et al. 2020; Eynard et al. 2020), which points towards a phenotypic expression altered by the respective environment. In line with this, heritability of recapping in uninfested and infested cells (RECall) was found to be low in a smaller Swiss population (Guichard et al. 2021). Apart from the study of Guichard et al. (2021) , other calculations of the heritability of targeted recapping of infested cells (RECinf) and RECall have so far been lacking for bigger populations, despite the fact that this factor is crucial for resistance breeding (Eynard et al. 2020; Hoppe et al. 2020).

1.2.4 Other factors leading to increased *Varroa*-resistance

In addition to the traits discussed in detail above, several other factors can add to the mechanisms of *Varroa*-resistance in honeybees. Besides differences in the virulence of different mite strains (haplotypes) and viruses (reviewed in [Rosenkranz et al. 2010]), these characteristics are mostly displayed on the individual or colony levels by the honeybees themselves. Among them, life-history traits like colony size, swarming, duration of brood breaks, or bee population development are most prominently linked to the overall adaptation of colonies to their respective habitat and therefore contribute to colony survival in more than one way (Locke 2016; Loftus et al. 2016; Locke et al. 2012). Smaller-sized brood cells have also been frequently assumed to suppress the reproduction of mites, though the effect of this seems to be limited (reviewed in [Le Conte et al. 2020]). In contrast, studies on grooming behaviour (i.e., worker bees remove mites from their own, or a nestmate's, body with their mandibles) have proved this trait to be relevant for the survival of several populations (reviewed in [Mondet et al. 2020a; Locke 2016; Rosenkranz et al. 2010]). Still, the measurement and application of this trait in breeding programs remains challenging (reviewed in [Rosenkranz et al. 2010]). Nevertheless, some breeding stocks appear to have been successfully selected for higher levels of grooming and correspondingly lower damage caused by *Varroa* (Morfin et al. 2020). Likewise, a shorter duration of the post-capping stage of brood was approached by targeted selection, since this trait was found to be heritable (Büchler & Drescher 1990) and distinctively expressed in some resistant populations (Locke 2016). In fact, shorter pupal development times in the capped brood cells decrease the number of

mature mite offspring in some populations, yet breeding efforts failed to select for this effect in managed populations (reviewed in [Le Conte et al. 2020]).

1.3 Thesis outline

Honeybees have evolved manifold resistance mechanisms against their principal parasite *Varroa destructor* (Mondet et al. 2020a; Traynor et al. 2020). This variety of resistance traits opens up a wide realm of studies in host-parasite interactions and their potential application in practical beekeeping and breeding. At the same time, the diversity of co-occurring traits greatly challenges comparable data acquisition for both, scientific investigations as well as bee breeding.

The frequent occurrence of MNR and REC in surviving colonies across the globe has made them candidate traits for targeted breeding efforts and thus urgent research topics (Mondet et al. 2020a; Oddie et al. 2018; Locke 2016).

My studies therefore focus on those traits to shed light on the underlying mechanisms up to the usability as selection criteria for breeding towards *Varroa*-resistance. This is urgently needed since several breeding programs already invest great efforts to select for both traits. However, up to now two crucial points have remained unknown: 1) whether those traits are heritable and thus selectable; and 2) whether recapping holds direct beneficial potential for the host colony.

My doctoral studies thus fill a knowledge gap and bridge basic research in resistance mechanisms with their application in practical science and breeding. I have, therefore, conducted extensive and long-lasting field trials to elucidate the interactions of different resistance traits and their applicability as selection criteria, as well as the effects of external factors.

At the outset of my studies, it was not known to which extent the expression of MNR and REC is genetically rooted or defined by external factors.

Since the presence of host brood reflects a key point for *Varroa*-reproduction, I first investigated brood interruptions as a possible external driver of reproductive failure in these mites (Chapter 2). The results gained by detailed dissemination of different forms of failed reproduction pointed towards a strong effect of brood interruptions on the occurrence of infertile mites as well as of REC. In contrast to my hypothesis that mite reproduction might be decreased only in brood cycles following the interruption, this effect was already visible during the interruption of brood rearing. The results thus indicated a dynamic change of bee behaviours and mite reproduction in response to external factors.

Next, I sampled related colonies in a common environment repeatedly over three years to further disentangle such outer effects on MNR and REC on a seasonal scale. This long-term investigation is presented in the third chapter (Chapter 3) of my thesis and has revealed significant seasonal variation in both traits. Despite seasonal variation, REC constantly showed a direct suppressing effect on mite reproduction by increasing levels of missing males and delaying the development of female offspring. As assumed, REC and MNR therefore proved to be valuable resistance traits, i.e., rewarding for selection. On the other hand, their phenotypic expression turned out to be greatly altered by external factors, which fits the results of Chapter 2 and complicates accurate measurements and selection decisions. Thus, the heritability of MNR and REC as the basis of selection was the logical next objective for study.

In Chapter 4, I present, for the first time, evidence for the heritability of both traits based on a comprehensive dataset of several thousand tested colonies. By compiling measured MNR and REC values with pedigree information, we were able to show that the traits are not only valuable, but also heritable and thus usable for targeted selection and breeding, as initially assumed. Hence, the results of Chapters 2, 3 and 4 all elucidate the underlying mechanisms of *Varroa*-resistance and lay the basis for future breeding efforts by improving the methods of performance testing and selection. In Chapter 5, I discuss the synopsis of the previous chapters in detail and conclude on their results. Furthermore, I point out implications for practical bee breeding, beekeeping and research and suggest important topics for future studies on this main pillar of sustainable apiculture.

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Chapter II

Immediate and long-term effects of induced brood interruptions on the reproductive success of *Varroa destructor*

II

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Original article

Immediate and long-term effects of induced brood interruptions on the reproductive success of *Varroa destructor*

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Abstract – The parasitic mite *Varroa destructor* (Anderson & Trueman) spends the dispersal phase of its life cycle on adult honeybees (*Apis mellifera* L.). The meaning of this phase for both bees and mites is still not well understood. This especially applies to prolonged dispersal phases as a result of brood interruptions. Hence, it is highly important to unravel this phase for understanding the underlying biological mechanisms and implementing this knowledge in beekeeping practice and research efforts. We investigated the effects of brood interruptions on honeybee colonies and the mites naturally infesting them. Reproduction parameters, brood infestation and recapping frequency were monitored over 60 days after brood interruptions of varying durations. Our results show that recapping frequency and mite non-reproduction increased during the interruption of egg laying. The duration of interruption and the time elapsed afterwards additionally affected the occurrence of reproductive failure. Hence, the reproduction of mites was affected by brood breaks immediately and in the long run.

Mite non-reproduction / Recapping / *Varroa* resistance / Biotechnical treatments / Brood breaks

1. INTRODUCTION

The ectoparasitic bee mite *Varroa destructor* is the major pathological threat for Western honeybees (*Apis mellifera*) and apiculture (Dietemann et al. 2012, 2013; Nazzi and Le Conte 2015; Rosenkranz et al. 2010; Vanengelsdorp et al. 2009). Many aspects of the delicate host-parasite relationship are well understood, since they have been studied intensively for decades (Nazzi and Le Conte 2015; Rosenkranz et al. 2010). However, large parts of the biology of the mite remain unclear. One example is the complex mating and reproduction biology of the mite, which plays a crucial role in population development and long-term colony survival (Fries and Rosenkranz 1996; Le Conte et al. 2020; Locke 2016; Otten 1991). The mite's life cycle comprises two phases: (1) a reproductive phase inside the brood cells and (2) a dispersal phase (often called "phoretic" in a broader sense) on adult honeybees (Traynor et al. 2020; Nazzi and Le Conte 2015; Rosenkranz et al. 2010). Both phases seem to be affected by various factors, which can lead to a suppressed reproductive success of the mites (Grindrod and Martin 2021; Locke 2016; Mondet et al. 2020a, b). The reproductive phase and invasion of brood cells have been studied intensively, giving insights into factors like brood type (Boot et al. 1992, 1995a, b; Fuchs 1990), olfactory cues (Frey et al. 2013; Garrido and Rosenkranz 2003; Rosenkranz and Garrido 2004), hygienic behaviour (Harris 2007; Mondet et al. 2016; Mondet et al. 2020a, b), intraspecific competition (Donzé et al. 1996;

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Donzé and Guerin 1994; Martin 1995b; Nazzi and Milani 1996) and duration of the post-capping period of brood cells (Büchler et al. 2010; Mondet et al. 2020a, b), which modulate the reproductive success of the mites. The dispersal phase has been studied less intensively, because mites are difficult to follow on adult bees at colony level (Fries and Rosenkranz 1996). Nevertheless, the dispersal phase between consecutive reproductive attempts likely plays a crucial role for the survival and reproduction of the parasite. Especially prolonged durations caused by brood breaks might affect the following reproductive phase.

Since researchers and breeders aim for comparable data by using mites of similar physiological and reproductive states, possible effects of previous brood breaks should be considered. For example, a lower reproductive success of mites was frequently reported for naturally surviving colonies (Grindrod and Martin 2021; Locke 2016; Oddie et al. 2018). Thus, this phenomenon is regarded as a selection criterion for breeding towards *Varroa* resistance (Büchler et al. 2010, 2020a, b; Mondet et al. 2020b), often measured after artificial infestation with mites gained from broodless donor colonies. Hence, such measurements on colony level might be distorted, if the expression of reproductive failure per se would be altered by brood interruptions. On the other hand, the same effects might be of special interest for beekeepers, particularly if the reproductive success of mites can be decreased. Though beneficial effects of swarm-related brood breaks on mite infestation of untreated colonies are known (Loftus et al. 2016; Seeley and Smith 2015; Fries et al. 2003), the infestation levels seem to be affected by multiple factors (Fries et al. 2003). Thus, the implementation of such brood breaks in practical beekeeping is usually combined with acaricide treatments (Büchler et al. 2020b).

Studies on the dispersal part of the life cycle of mites have so far mainly focused on host preferences in terms of age and task of the adult bees parasitized (Cervo et al. 2014; Xie et al. 2016) or invasion behaviour (Beetsma et al. 1999). Though host preference may change with infestation on colony level (Cervo et al. 2014), mites prefer nurse bees as adult hosts over foragers and freshly emerged bees. This preference also corresponds to a better reproductive success of mites previously parasitizing nurse bees as adult hosts (Xie et al. 2016). Likewise, Stürmer and Rosenkranz (1994) reported a higher reproductive success of mites formerly parasitizing in colonies containing nurse bees (i.e. colonies with open brood) in comparison to mites spending their dispersal phase in colonies without brood and nurse bees. The reproductive success of these mites was decreased after artificially prolonged dispersal phases of up to 12 weeks in broodless colonies (Stürmer and Rosenkranz 1994). While no effect of the duration of naturally chosen dispersal phases was reported (Boot et al. 1995a, b; Piou et al. 2016), these findings indicate that the reproductive success of mites can be artificially altered depending on the duration of the previous dispersal phase. Such a possible effect of brood interruption is crucial for (1) bee breeding and (2) science in which mites of comparable states are needed for various bioassays respectively (Dietemann et al. 2013), as well as (3) practical beekeeping in which brood interruption methods are valued for *Varroa* control (Büchler et al. 2020b).

Hence, we here investigated immediate and long-term effects of induced brood interruptions of different durations on the reproductive success of *Varroa destructor* on colony level.

2. MATERIALS AND METHODS

Experiments were conducted in the summer of 2019 at the LLH Bee Institute Kirchhain (Hesse, Germany) with 27 full-grown colonies headed by open mated queens derived from different mothers of the Institute's Carniolan breeding stock.

Figure 1. Schematic overview over sampling dates of brood combs. Queens were caged for 10, 20, or 30 days to induce a brood interruption of corresponding duration or were left unrestricted as a control group. Arrow symbols (→) indicate the first sample set at the beginning of the study (i.e., during caging), following samples are marked with (1), (2), or (3) to indicate the first, second and third brood cycles sampled after caging.

All colonies were lodged in hives comparable to two Langstroth standard boxes and placed at the same apiary, while replicates belonging to the respective experimental groups were distributed randomly over the location. Queens were either caged in mid-July for 10, 20, or 30 days ($n = 7$ each) to induce an interruption of egg laying of corresponding duration or were left uncaged as a control ($n = 6$). For queen caging, standard cages with queen excluder sidewalls were used as described by Büchler et al. (2020b).

2.1. Sampling

Brood combs of treatment groups were subsequently sampled for brood investigation at four time points: (1) while queens were caged (10 days after caging), (2) in the first supposed brood cycle of mites after caging, (3) in the second supposed brood cycle of mites after caging and (4) in the third supposed brood cycle of mites after caging (Figure 1). Thus, the first set of brood combs (1) was sampled at the same date in all treatment groups (Figure 1). The following three sampling dates after the release of the queens (2nd, 3rd and 4th sets of brood combs) differed according to the duration of caging in the respective groups (Figure 1). Irrespective of the date, the subsequent brood combs were sampled in 20 days intervals (i.e., 2nd, 20 days; 3rd, 40 days; and 4th, 60 days) after the release date of the queens respectively (Figure 1). This timing enabled investigations on the reproductive success of mites according to Büchler et al. (2017), since most mites perform a dispersal phase of approximately 7 days before invading a cell in the L5 larval stage (Boot et al. 1993; Harbo and Harris 1999; Rosenkranz et al. 2010; Sammataro et al. 2000) and thus were found in a suitable brood age for investigation (i.e., 7–12 days post capping) of reproductive parameters after 20 days. Though the date of cell-invasion can only be extrapolated due to variation in individual mites' behaviour, the time frame of 7 days post capping up to emergence of the bee allows for some flexibility in the investigation of reproductive success (Büchler et al. 2017). Additionally, brood combs of untreated control

colonies with constant brood rearing activity were sampled. This sampling was performed four times to account for possible seasonal variation (Otten 1991) while avoiding an oversampling (i.e., weakening) of control colonies (Figure 1). These control samples were distributed over the course of the study to keep the time span between samplings as short as possible (Figure 1), since differences between long-term measurements of mite reproduction were found to be higher than in measurements in quick succession (Eynard et al. 2020).

Importantly, young mites from non-sampled brood combs were expected to hatch in 20-day intervals during the study (Harbo and Harris 1999), while mites inside the sampled brood combs were lethally removed for investigation of reproductive success. Hence, the sampling dates for the supposed brood cycle of mites after caging refer to the whole mite population in the hive instead of individual mites.

2.2. Brood investigation

All brood samples were stored at – 20 °C until investigation. Overall, 19,084 brood cells (7–12 days post capping) were investigated with respect to their proportionate infestation with mites (i.e., brood infestation) as well as the occurrence of mite non-reproduction (MNR) and recapping (REC) in single infested cells ($n = 2579$). Brood infestation rates were automatically calculated during the brood investigations. Investigations followed the protocol of the Research Network on Sustainable Bee Breeding (Büchler et al. 2017), more recently also described in Büchler et al. (2020a, b). Accordingly, reproductive failure in terms of MNR was defined by a mother mite solely infesting a cell with either no offspring (infertile), only female offspring (no male) or progeny which was too young in comparison to the developmental stage of the respective host bee pupae (delayed).

2.3. Statistical analysis

All statistical analyses were conducted in the R environment (version 4.1.0, R Core Team 2021). Generalized linear mixed-effect models (*glmer*) from the binomial family (*logit*) were used to estimate the probabilities of recapping and non-reproduction on cell level (Bates et al. 2015). The occurrence of recapped cells and non-reproductive mites (including different types of failed reproduction) was considered a response variable. Treatment (i.e., duration of caging) and brood cycle after caging (i.e., subsequent samplings) were implemented as fixed explanatory variables including interactions. In case of non-reproduction and different types of reproductive failure, recapping did overall not contribute to an improved prediction accuracy and was therefore not treated as another explanatory variable. However, this was not the case in a subset of data gained from the first set of brood combs (during caging, Figure 1). In this subset, recapping was included as an explanatory variable alongside with treatment (caged or control) and the respective interactions to investigate the effects on mite reproduction. Tested colonies were considered separate mite populations and thus included as a random factor. Residuals and over-dispersion were analysed using the *DHARMa* package (Hartig 2021). Subsequent pairwise comparisons among factor levels were performed using Tukey post hoc tests (*emmeans* (Lenth 2021)).

Due to the data structure, a beta regression (*betareg* (Cribari-Neto and Zeileis 2010)) was calculated alongside with the functions *lrtest* (*lmtest* (Zeileis and Hothorn 2002)) and *joint_tests* (*emmeans* (Lenth 2021)) in case of brood infestation. Fixed and random factors were implemented in this model as described above.

3. RESULTS

3.1. Mite non-reproduction (MNR)

On individual cell level, the predicted probability of MNR was significantly affected by the duration of brood interruption (GLMM: $\chi 2 = 30.4$, $p < 0.001$, $df = 3$). Overall, the probability of reproductive failure increased with the duration of caging. Longer caging durations also seemed to induce a longer-lasting increase in

MNR in the treated colonies compared to control colonies with constant brood rearing (Figure 2).

There was no clear effect of brood cycle after caging alone. However, we found an interaction effect of brood cycle after queen caging and duration of brood interruption (GLMM: $\chi 2 = 17.52$, $p = 0.04$, df = 9). We therefore report differences between treatment groups separately for each sampling time.

While queens were still caged, the predicted probabilities of MNR in all three groups with brood interruption were significantly higher than in control colonies with undisturbed brood activity, demonstrating a strongly suppressing immediate effect of brood interruption on mite reproduction ($p < 0.001$ each, Figure 2). Treatment groups with caged queens did not differ in the probability of reproductive failure of mites among each other, while queens were still caged.

In the first brood cycle after caging, probabilities of reproductive failure did not differ between the treatment groups but still tended to be higher in comparison to the control group with continuous brood activity (Figure 2). Here, the highest probabilities of reproductive failure were predicted at this sampling point compared to the other sampling dates within this group (Figure 2).

In the second brood cycle after caging, probabilities of failed reproduction were notably higher in the group with 30 days of brood interruption, which differed significantly from the control group ($p < 0.05$). Though no other statistically proofed differences between treatment groups were found, probabilities of failed

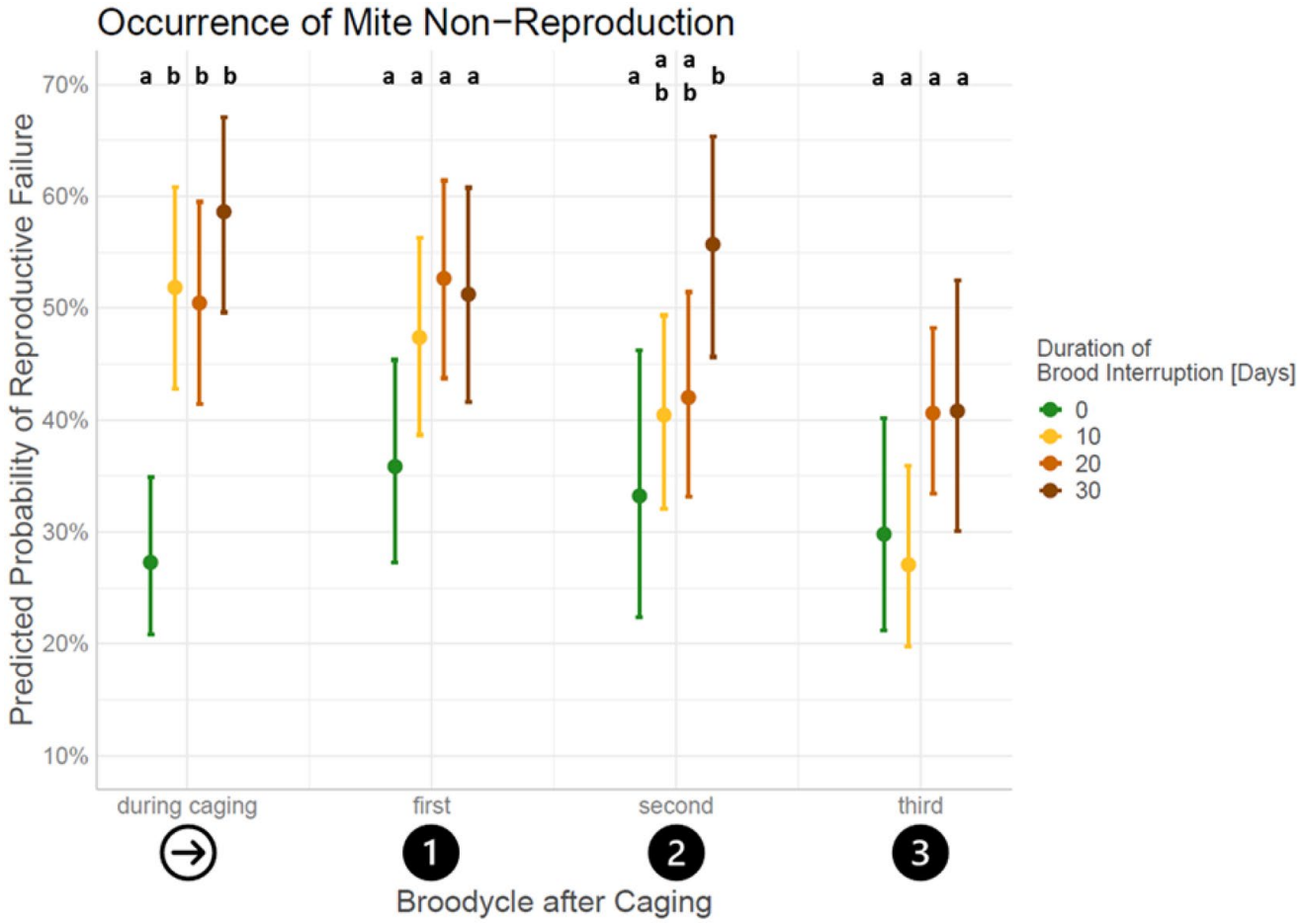
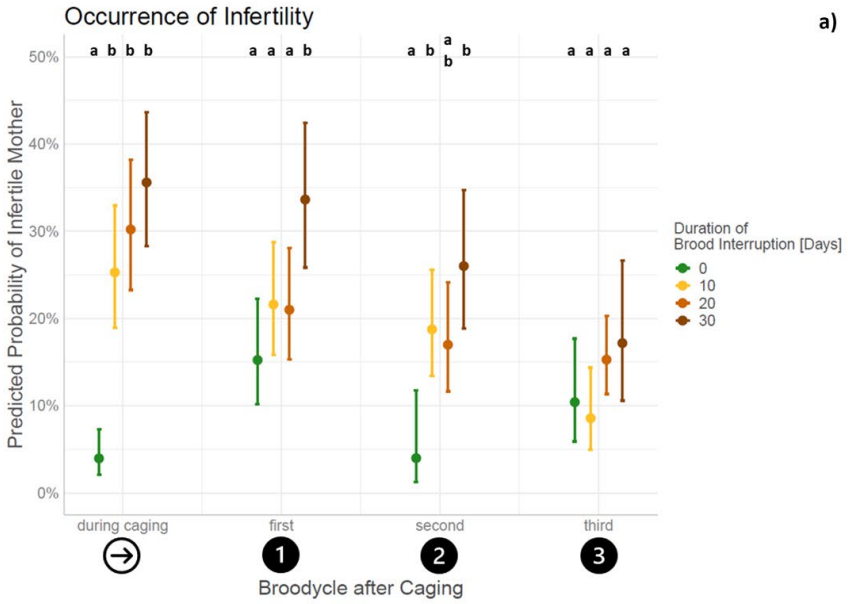
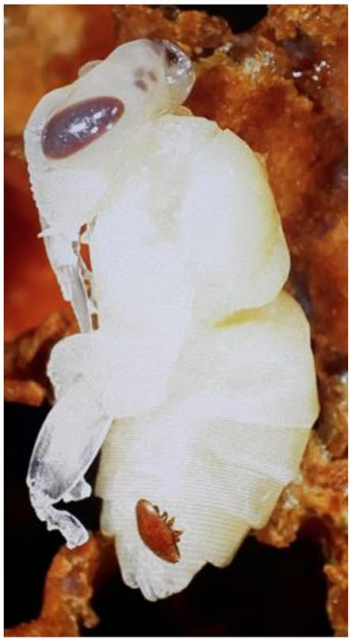
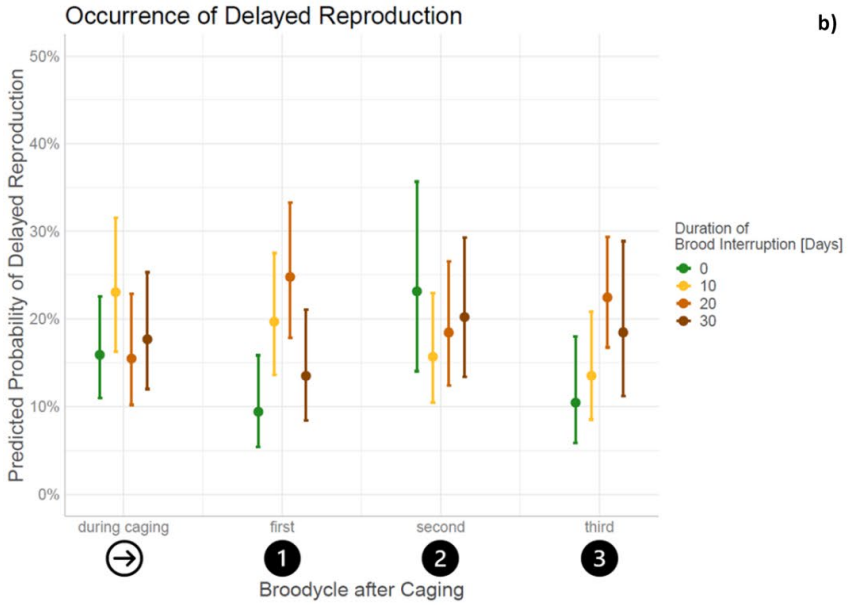


Figure 2. Predicted probabilities of reproductive failure (MNR) of mother mites in brood combs sampled over the study period (displayed with 95% CI). Symbols below the *x*-axis refer to the sampling points marked in Figure 1. The duration of 0 days of brood interruption (green symbols) corresponds to the unrestrained control group. The probability of reproductive failure was significantly influenced by the duration of brood interruption (GLMM: $\chi 2 = 30.4$, $p < 0.001$, df = 3) and an interaction of the duration of brood interruption and brood cycle (GLMM: $\chi 2 = 17.52$, $p = 0.04$, df = 9). Different letters indicate significant differences (Tukey HSD, $p < 0.05$ each) among treatment groups within the respective brood cycle after caging.

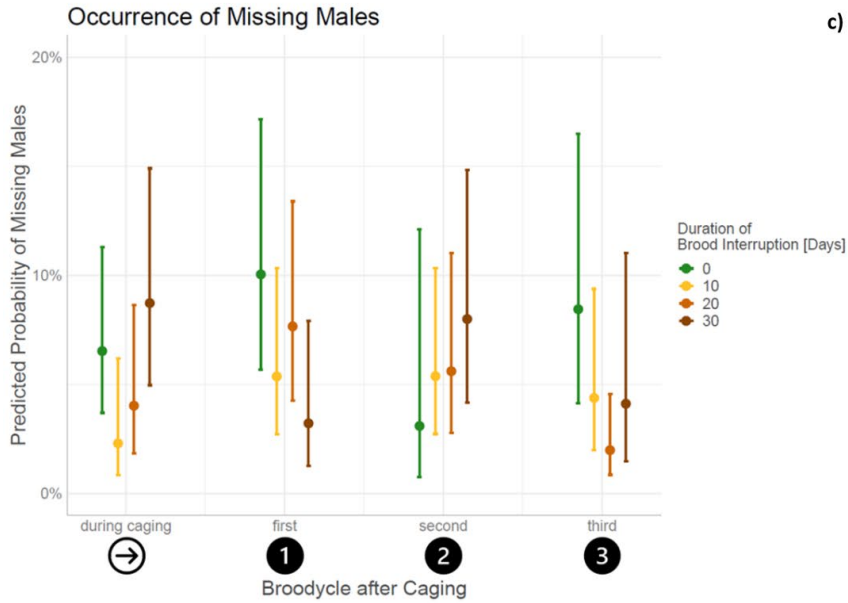

Occurrence of Infertility
a b b b
a a a b
a b a b
a a a a
50%
40%
30%
20%
10%
0%
Predicted Probability of Infertile Mother
during caging
first
second
third
1
2
3
Broodycle after Caging
Duration of
Brood Interruption [Days]
0
10
20
30

a)




Occurrence of Delayed Reproduction
50%
40%
30%
20%
10%
0%
Predicted Probability of Delayed Reproduction
during caging
first
second
third
1
2
3
Broodycle after Caging
Duration of
Brood Interruption [Days]
0
10
20
30

b)


Occurrence of Missing Males
20%
10%
0%
Predicted Probability of Missing Males
during caging
first
second
third
1
2
3
Broodycle after Caging
Duration of
Brood Interruption [Days]
0
10
20
30

c)

◂**Figure 3.** Predicted probabilities of **a** infertile mother mites, **b** delayed reproduction and **c** missing males in brood combs sampled over the study period (displayed with 95% CI). Pictures showing the respective cause of reproductive failure (**a**)–(**c**) in brood cells approximately 9 days post capping: **a** infertile mother, **b** delayed reproducing mother and male, **c** mother and deutonymph daughters without male. Symbols below the *x*-axis refer to the sampling points marked in Figure 1. The duration of 0 days of brood interruption (green symbols) corresponds to the unrestrained control group. Different letters indicate significant differences (Tukey HSD, $p < 0.05$ each) among treatment groups within the respective brood cycle after caging. Further test statistics are given in the text.

reproduction seemed to be staggered according to the duration of previous brood interruption in the respective groups (Figure 2).

Though the treatment groups with 20 and 30 days of brood interruption still tended to show the highest values in the third brood cycle after caging, predicted probabilities of failed reproduction seemed to be in a comparable range among all groups at this time (Figure 2).

To investigate the immediate effect found during queen caging, colonies with caged queens (i.e., groups 10, 20 and 30) were additionally compared as one treatment group with the uncaged control group at this point in time. Notably, at this date, the caged queens of all treatment groups were restricted in egg laying for the same duration (i.e., 10 days; Figure 1). The predicted probability of MNR in this subset of data was likewise affected by treatment as described above with distinctively higher values in the treatment group (GLMM: $\chi 2 = 15.2$, $p < 0.001$, $df = 1$). Neither in treated colonies nor in untreated colonies the recapping status of cells showed a significant effect on the occurrence of MNR. However, MNR values tended to be higher in untouched cells respectively (Fig. 1 in supplements).

3.2. Cause of reproductive failure

The underlying causes of MNR (infertile mother, delayed reproduction or missing male) were examined separately. Interestingly, they seemed to be affected differently by the factors investigated (Figure 3). Neither occurrence of missing males nor delayed reproduction seemed to follow a specific pattern related to the duration of brood interruption or the brood cycle sampled (Figure 3b, c).

In contrast to these failures in fertile mites (missing males or delayed reproduction), the predicted probability of infertile mother mites was overall strongly affected by the duration of brood interruption (GLMM: $\chi 2 = 50.29$, $p < 0.001$, $df = 3$). Similar to the general pattern of MNR, longer durations of brood interruption seemed to have a stronger and longer-lasting effect on the occurrence of infertility (Figure 3a). The probability of infertile mother mites was also affected by the brood cycle sampled (GLMM: $\chi 2 = 16.59$, $p < 0.001$, $df = 3$) and an interaction of treatment and sampling time (GLMM: $\chi 2 = 28.35$, $p < 0.001$, $df = 9$). Therefore, pairwise comparisons between treatment groups are reported separately for each sampling time. The probability of infertile mites was remarkably higher in all groups with brood interruption in comparison to the unrestricted control group, while queens were still caged ($p < 0.001$ each, Figure 3a). In the following brood cycles, the probability of infertile mothers in treatment groups decreased gradually towards that found in the control group. This became particularly apparent when the brood interruption lasted for 30 days (Figure 3a). The probability of infertile mothers in this group decreased in the first and second brood cycles after caging, but was still significantly higher in comparison to the control group ($p = 0.004$ respectively, Figure 3a). The same trend appeared in the first and second brood cycles after caging for the groups previously restricted in brood rearing for 10 and 20 days (Figure 3a). By the time of the third assumed brood cycle after caging, predicted probabilities for infertility did not differ significantly between groups, but still tended to be higher in the group formerly caged for 30 days (Figure 3a).

As described above for MNR, the underlying causes of reproductive failure were also investigated between the treated and untreated colonies by the time of queen caging. Likewise to the general occurrence of infertile mothers, the

probability of infertility in mites at the beginning of the study was strongly affected by treatment (GLMM: $\chi2 = 20.76$, $p < 0.001$, df = 1) with distinctively higher values in colonies with caged queens (Fig. 2 in supplements). Although the recapping status of cells (GLMM: $\chi2 = 4.32$, $p = 0.04$, df = 1) as well as the interaction of recapping and treatment (GLMM: $\chi2 = 4.08$, $p = 0.04$, df = 1) proofed to have a significant effect on the occurrence of infertile mites, this effect seemed to be limited to the uncaged control group. In control colonies, the probability of infertile mites was significantly increased in recapped cells ($p < 0.04$), while recapping showed no effect on the overall high values of the treatment group (Fig. 2 in supplements). In contrast, the occurrence of delayed reproduction or the absence of males was neither affected by the recapping status of cells, nor the treatment of the respective colony. The probability for delayed reproduction tended to be higher in colonies with caged queens and in untouched cells, while there was no obvious trend in the absence of males (Figs. 3 and 4 in supplements).

3.3. Recapping (REC)

The predicted probability for recapping on individual cell level was significantly affected by treatment (GLMM: $\chi2 = 9.41$, $p = 0.02$, df = 3). This also applied for the brood cycle sampled (GLMM: $\chi2 = 9.41$, $p < 0.001$, df = 3), as well as an interaction of these factors (GLMM: $\chi2 =$

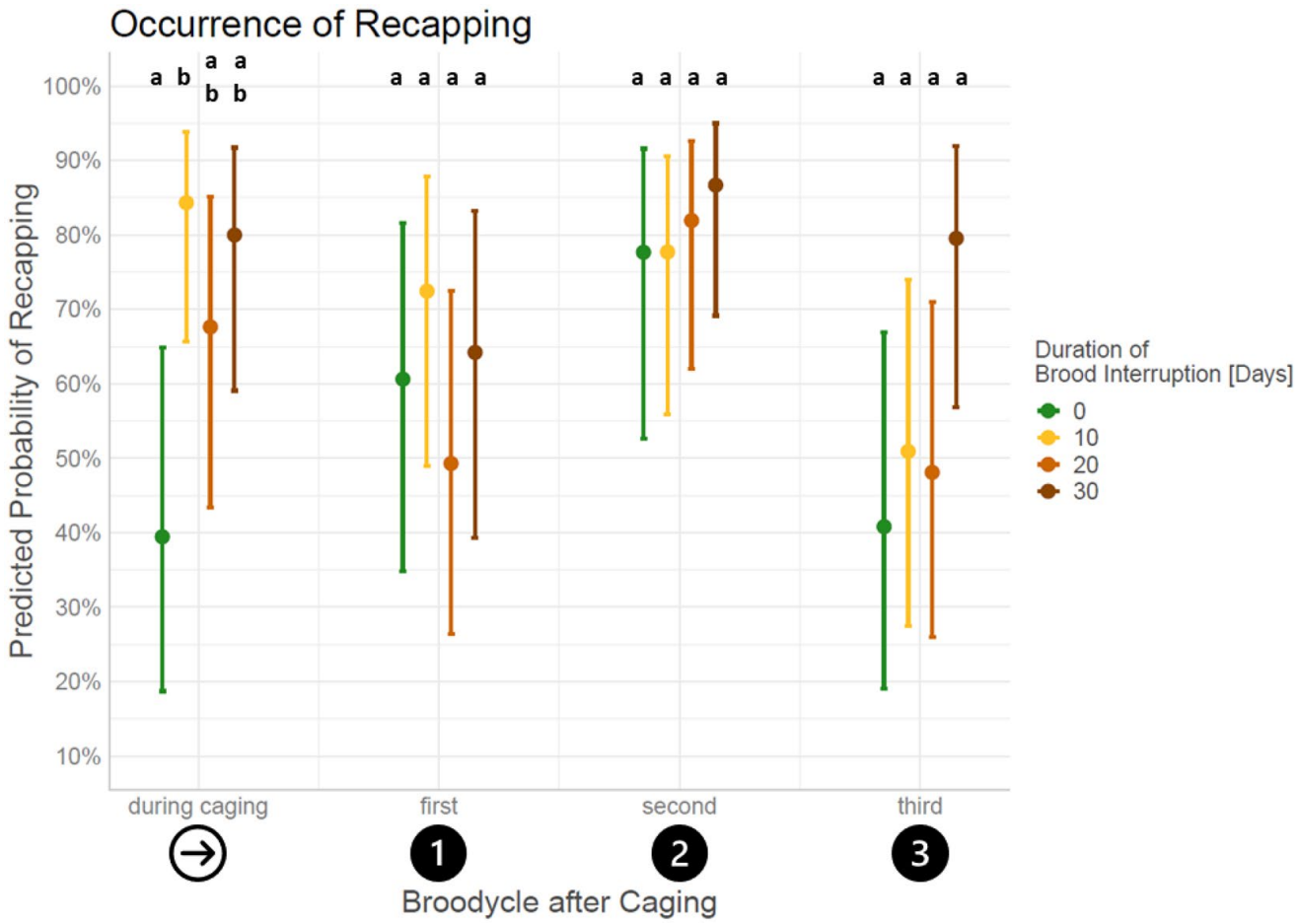


Figure 4. Predicted probabilities of recapping (REC) in brood combs sampled over the study period (displayed with 95% CI). Symbols below the *x*-axis refer to the sampling points marked in Figure 1. The duration of 0 days of brood interruption (green symbols) corresponds to the unrestrained control group. The probability of recapping was significantly influenced by the duration of brood interruption (GLMM: $\chi2 = 9.41$, $p = 0.02$, df = 3), the brood cycle sampled (GLMM: $\chi2 = 9.41$, $p < 0.001$, df = 3), as well as an interaction of these factors (GLMM: $\chi2 = 63.5$, $p < 0.001$, df = 9). Different letters indicate significant differences (Tukey HSD, $p < 0.05$ each) among treatment groups within the respective brood cycle after caging

63.5, $p < 0.001$, df = 9). However, differences between groups were only visible while queens were caged in the treatment groups (Figure 4). At this time, all treatment groups with caged queens displayed higher predicted probabilities of recapping than the control group with unrestricted brood rearing. Although this trend was only statistically proven in one of the three caging groups when analysed separately (group 10: $p = 0.03$, Figure 4), the same effect was generally found when comparing all treated colonies against the control group as described above (GLMM: $\chi 2 = 8.09$, $p = 0.005$, df = 1, Fig. 5 in supplements). In the following brood cycles after caging, predicted probabilities of recapping varied largely within groups and were lacking a clear pattern over time.

3.4. Brood infestation

The percentage of infested brood cells was significantly affected by treatment (GLMM: $F = 27.42$, $p < 0.001$) with overall lower infestation levels in colonies which experienced a brood interruption (Figure 5). The infestation level was also affected by the time of sampling (GLMM: $F = 3.02$, $p = 0.03$) and an interaction between both of these factors (GLMM: $F = 3.2$, $p < 0.001$). Over the course of the study, this

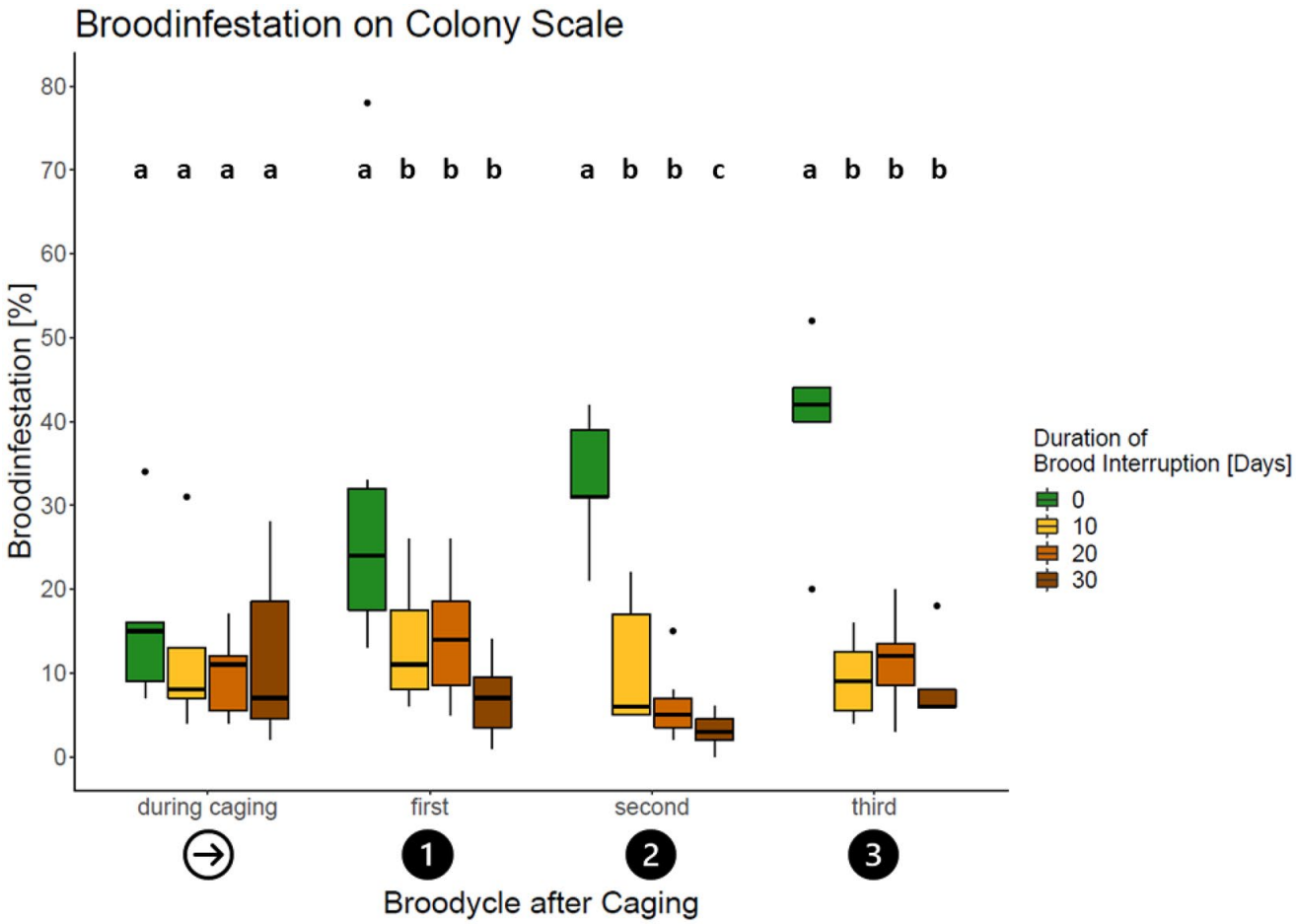


Figure 5. Brood infestation of investigated colonies over the study period. Boxplots display median values (inner horizontal lines), 1st and 3rd quartiles (box), minimum and maximum values (whiskers) and outliers (filled dots). Symbols below the *x*-axis refer to the sampling points marked in Figure 1. The duration of 0 days of brood interruption (green symbols) corresponds to the unrestrained control group. The brood infestation was significantly affected by the duration of brood interruption (GLMM: $F = 27.42$; $p < 0.001$), the brood cycle sampled (GLMM: $F = 3.02$; $p = 0.03$), as well as an interaction of these factors (GLMM: $F = 3.2$; $p < 0.001$). Different letters above boxplots indicate significant differences (Tukey HSD, $p < 0.05$ each) among treatment groups within the respective brood cycle after caging.

effect was displayed by a constantly increasing brood infestation in the control group (Figure 5). In contrast, groups which experienced a brood interruption before generally showed little variation in the following brood cycles (Figure 5). Notably, brood infestation levels did not differ between groups at the beginning of the study (i.e., first sampling, Figures. 1 and 5). This already changed by the time of the first brood cycle after caging. At this time, control colonies with previously unrestricted brood rearing showed much higher brood infestation levels compared to all treatment groups ($p < 0.05$ each), which did not differ from each other (Figure 5). The infestation level in control colonies further increased across the second and third brood cycles after caging, while there was no visible increase in the brood infestation of colonies with previous brood interruption (Figure 5). Thus, at the end of the study (i.e., third brood cycle after caging, Figure 1), the brood infestation in all three treatment groups was significantly lower compared to that in the control group with unrestricted brood rearing ($p < 0.001$ each, Figure 5).

4. DISCUSSION

4.1. Brood interruption reduces reproductive success of mites

Our experiments challenged the hypothesis that brood interruption can alter the reproduction of *Varroa destructor*.

Honey bee brood is crucial for mite reproduction (Martin 1995a). Thus, brood interruptions, e.g., as a consequence of swarming, seem to be an obstacle for mite reproduction per se. Our results demonstrate that brood interruptions suppress the reproductive success of mites beyond the mere temporary lack of opportunities for cell invasion. Notably, the share of reproductive failure over all treatment groups was highest while the queens were still caged. The mites sampled at this point were suspected to have entered suitable brood cells shortly before the queens' egg laying stopped. Thus, the observed decrease of reproductive success during brood interruption cannot be explained by a prolonged dispersal phase. Among the known traits associated with increased MNR on colony level, REC is one of the most frequently found behaviours (Grindrod and Martin 2021; Mondet et al. 2020a, b). However, the exact mode of action is still unknown. Natural REC on cell level does not seem to interact directly with the reproductive success of mites infesting the respective cells (Martin et al. 2020; Oddie et al. 2018; Harris et al. 2012; Martin et al. 1997), which overall corresponds with the present results.

Given the complex mating biology of mites in dependence to the honeybee host, it is likely that the importance of single resistance traits also varies over time, e.g., due to seasonal variations in brood rearing or intensity patterns of other worker bee duties. Tison et al. (2022) just recently showed that the *Varroa*-sensitive hygiene behaviour (VSH) can be less pronounced as a result of increased foraging during strong nectar flows. In our case, the decreasing demand for larvae feeding might have favoured the distinctively increased REC observed during brood interruption in all treatment groups. Although we found no direct effect of REC on MNR, the increase of MNR could be similarly explained by an increased removal of infested brood cells (VSH), as Martin et al. (2020) described REC as a valuable and closely linked proxy for VSH. Though VSH was not investigated in the present study, the observed trend of higher MNR values in untouched (i.e., not recapped) cells additionally supports this hypothesis. The removal of infested brood cells was repeatedly supposed to be biased towards reproductive mites, leading to an increased proportion of MNR in the remaining cells (summarized in Mondet et al. 2020a, b; Oddie et al. 2018).

However, the selective removal of reproductive mites was not confirmed in other studies on VSH (Sprau et al. 2021; Harris et al. 2010), underlining the variability and complexity of linkages between resistance traits. Thus, the occurrence of MNR is most likely affected by a diverse set of traits and interactions varying over time. Although we can only speculate about the underlying mechanisms leading to the

spontaneous increased MNR values in the treatment groups, the interruption of brood activity as the initial cause is clearly proven.

The present results also demonstrate long-term effects on MNR. Brood interruptions and correspondingly prolonged dispersal phases in summertime appear to reduce the success of mites' in following reproductive attempts. Similar effects have been shown for natural winter brood breaks by Otten (1991, see also Otten and Fuchs 1990), as well as for artificially prolonged dispersal phases in summertime (Stürmer and Rosenkranz 1994). In the present study, the suppressing effect on mite reproduction was still visible when the new brood nest comprised all larval and pupal stages again. However, the differences in reproductive success of mite populations in formerly treated and control colonies decreased over time. By the time of the third brood cycle after caging, all treatment and control colonies showed similar MNR values. This recovery effect on population scale fits well to the described number of reproductive cycles for individual *Varroa* females on colony level, since mites are assumed to reproduce two to three times in a row (Martin and Kemp 1997; Fries and Rosenkranz 1996). Therefore, the gradual recovery of mite reproduction on colony level might be explained by the substitution of old mites (which experienced the brood interruption) by young mites (which hatched afterwards). Likewise, the mite reproduction recovered more quickly after shorter brood interruptions since the proportion of mites forced into a prolonged dispersal phase was correspondingly lower. In addition, the time of sampling showed no direct effect on MNR but significant interactions with the treatment, pointing towards a change in mite population structure rather than a general temporal variability of MNR.

Hence, brood interruptions and prolonged dispersal phases add to various other causes like brood cues and behaviours of adult bees (Mondet et al. 2020a, b) which can alter the reproductive success of mites. Especially the duration of the dispersal phase appears to be important for following reproductive attempts. In fact, the exact role of this part of the mites' life cycle is still unknown (Rosenkranz et al. 2010; Xie et al. 2016). Early studies showed that mites are able to reproduce up to seven times in a row without a dispersal phase in between (de Ruijter 1987). However, in the first reproductive attempt, this applies most probably only for the oldest of the freshly hatched daughters which already completed the spermatozoa capacitation (Häußermann et al. 2016). Obviously, the dispersal phase in summertime harbours some benefits for the mites since the divided life cycle evolved as an alternative to direct transition into the next reproductive attempt. For example, transportation by the host bees enables the mites to reach new brood cells in both, the current colony by using nurse bees, as well as non-natal colonies by attaching to drifting or robbing foragers (Frey and Rosenkranz 2014; Nazzi and Le Conte 2015; Peck and Seeley 2019). On the other hand, it may also pose dangers for the mites (Pritchard 2016; Rosenkranz et al. 2010; Xie et al. 2016) and is not obligatory for successful reproduction in every case (de Ruijter 1987; Häußermann et al. 2016). Our results show that a decrease in reproductive success on colony level can add to these previously described negative effects for the mites if the dispersal phase is prolonged.

These effects of brood interruptions and corresponding dispersal phase durations should be taken into account in different contexts. The reproductive success of the mite population on colony level holds great importance for the overall infestation and thus the ultimately survival chances of a colony (Rosenkranz et al. 2010). Notably, the here tested durations of 10, 20 and 30 days of brood interruption are field-realistic time spans in naturally swarming colonies. After settling in a new location, it takes at least 10 days for the swarm to produce brood cells old enough for mite invasion (Rosenkranz et al. 2010; Winston 1987). In turn, the remaining part of the colony usually needs between 22 and 30 days after swarming until the young queen starts egg laying (Koeniger et al. 2014; Seeley and Smith 2015; Winston 1987). In this light, natural brood interruptions are usually rated as beneficial for infested colonies (Loftus et al. 2016). Our results suggest that the duration of such swarm-associated brood breaks may also affect mite population

development and thus general health status in both parts of the swarmed colony.

Likewise, induced brood interruptions used in beekeeping (Büchler et al. 2020b) may also hold a potential for biotechnical treatments against *V. destructor* even without a subsequent drug application.

In addition to these implications for practical beekeeping, immediate and long-term effects should be taken into account whenever gathering mites for bioassays used in bee breeding or science. This is done mainly by caging queens for brood interruption in highly infested "mite-donor-colonies" in order to force mites into a dispersal phase in which they can be detached easily from the bees by powdered sugar-shakes (Dietemann et al. 2013). Hence, the afterwards investigated reproduction of mites could be altered by the previously induced brood break. However, in the present study, this effect was less expressed in colonies with shorter durations of queen caging and decreased over time. Therefore, shorter durations of brood interruption in the "mite-donor-colonies" as well as a recovery-phase for the mite population could compensate for the effects of brood interruption when working with artificially infested colonies.

4.2. Brood interruption affects causes of reproductive failure differently

Overall, failed reproduction of mites is characterized by (1) the lack of male offspring; (2) delayed oviposition, desynchronizing age of mite offspring and developmental stage of the host cell; or (3) infertile mother mites. The present results indicate that brood interruptions alter the proportional occurrence of factors causing reproductive failure of mites. Infertility was the most common cause for reproductive failure of mites in treatment groups (48%). It was followed by delayed reproduction (41%) and missing males (11%). This is in contrast to earlier findings by Mondet et al. (2020b) in colonies undisturbed brood activity. By comparing the putative causes of reproductive failure of mites in 106 colonies from six different countries, delayed reproduction was found to be the most common cause (Mondet et al. 2020b). It was followed by infertile mites and mite families without males, while the composition of the respective causes differed significantly between locations (Mondet et al. 2020b). The occurrence of infertility, delayed reproduction and missing males in our control colonies resembled the previously described values (Mondet et al. 2020b). Thus, the differing distribution in treatment groups of the present study seemed to be rather an effect of the brood interruptions than of the location.

Interestingly, the probability for infertile mites was remarkably higher during caging in treatment groups compared to untreated control groups in the present study. Over the course of the subsequent samplings, it converged with those of the control group. It thus showed a similar pattern as the overall reproductive success of mites. Our results strongly indicate that brood activity forms one of the mechanisms affecting the proportion of infertile mother mites. In addition to this effect of brood activity on colony level, the effect of honeybee brood signals on the fertility and reproductive success of mites has been shown for age-related factors inside individual host cells (Frey et al. 2013; Kirrane et al. 2011; Sprau et al. 2021). Those studies showed that the right host age is crucial for oogenesis and proper timing of egg laying in fertile mites. Interestingly, the occurrence of infertile mites, i.e., the absence of egg laying was shown to be higher if mother mites were artificially transferred into older brood cells, even if they already started oogenesis before (Frey et al. 2013). Such transfer situations (from brood cell to brood cell) or mistimed invasions (from dispersal phase to brood cell) could occur under natural circumstances due to resistance behaviours of the bees. Especially, VSH (Kirrane et al. 2011; Mondet et al. 2020a, b) and REC (Grindrod and Martin 2021; Oddie et al. 2018) could potentially lead to such mismatches between host-age and the reproductive status of mites. In addition, the standard method of brood investigation on frozen brood combs, as used in this study, is not capable of the differentiation between mites which already died before sampling from those which were alive at the time of sampling. Thus, some of the non-reproductive mites may simply lacked

proper offspring because they died shortly after cell invasion, e.g., as a consequence of recapping. Both hypotheses would explain the significantly higher probabilities of infertile mites in recapped cells of the control group compared to untouched (i.e., not recapped cells) of these colonies. Interestingly, such differences were not found during brood interruption in the treated colonies. In the treatment group, the probability of infertile mothers was overall high in recapped as well as untouched cells. Again, this might be the outcome of selective VSH towards reproducing mites as a consequence of task allocation as discussed in detail in Sect. 4.1.

Although the exact mechanisms leading to different causes of reproductive failure can only be hypothesized, the duration of brood interruption and the time elapsed after caging clearly affected the occurrence of infertile mothers. In addition to these colony level factors, REC altered the occurrence of infertile mothers on cell level at least in some cases. However, the probability for missing males and delayed reproduction appeared to be mostly unaffected by REC and the brood interruptions investigated in the present study.

This again underlines the complexity of the host-parasite interactions between mites and bees as well as the need for further studies on the underlying mechanisms of reproductive failure in mites.

4.3. Queen caging temporarily increases recapping

The uncapping of sealed brood and subsequent recapping of the cells by worker bees (REC) is a common trait in naturally *Varroa*-surviving honeybee populations (Grindrod and Martin 2021). It also occurs in *Varroa*-naïve colonies, albeit to a lower degree (Martin et al. 2020). Our results indicate that REC on colony level, likewise to the reproductive success of mites, is also affected by brood interruptions. Notably, the frequency of REC was highest during the caging of queens in treatment groups, which corresponds to a higher probability of reproductive failure of mites at this time. However, in contrast to our findings on mite reproduction, the effect of brood interruptions on REC was only visible during the restriction of egg laying. Thus, it might be a direct but short-term behavioural reaction of the bees to the changing relation of adult bees to young brood cells as shown for other worker duties before (Tison et al. 2022). Nevertheless, this temporal effect on the behaviour of bees might have contributed to a lower reproductive success of mites, both directly as well as in later reproductive attempts as discussed above.

Although REC overall did not show a statistically significant effect on MNR, this could have been masked by comparatively stronger effects of the treatment and sampling time, as supposed for other parameters co-occurring with REC (Oddie et al. 2021) and discussed in Sect. 4.1.

This also corresponds to higher MNR values, which were found in artificially, but not in naturally recapped cells (Oddie et al. 2018). However, *Varroa*-surviving honeybee populations frequently display higher levels of MNR than susceptible colonies (Grindrod and Martin 2021; Locke 2016), which was recently shown to be directly affected by the recapping frequency of infested cells on colony level (Oddie et al. 2021). This likewise points to a more complex effect of REC on MNR, which may be sometimes hidden on colony level.

Overall, the mite depressing effects of REC are increasingly gaining attention, promoting this trait as an appropriate criterion for selection towards *Varroa*-resistance (Büchler et al. 2020a, b; Oddie et al. 2021). The present results show that the expression of this trait is also linked to brood interruptions in the colonies investigated, which should be taken into account when measuring recapping rates. Since there was no clear pattern in the occurrence of REC over the course of subsequent samples after caging, it is likely that the behaviour of individual colonies was altered additionally by other environmental factors (Oddie et al. 2021).

4.4. Long-lasting reduction of brood infestation after brood interruption

Over the course of the study, brood interruptions had a clear effect on infestation levels of worker brood. Infestation levels were found to be high (on average 12%) but comparable among

treatment groups and the untreated control colonies in the beginning of the study. Although the infestation level of control colonies tended to be higher during the caging of queens in the treatment groups, the mite loads did not differ significantly between groups at this time. In contrast, mite loads were remarkably higher in the control group compared to the three treatment groups already by the time of the first brood cycle after caging. Consequently, this difference increased continuously until the end of the study. This was partly expected due to the interrupted mite population growth in treatment groups, in contrast to the continuous brood activity in the also highly infested control group. Similar differences in infestation levels are known to be of great importance for the health status of naturally swarming colonies (Loftus et al. 2016; Seeley and Smith 2015). Nevertheless, the higher reproductive success of mites with continuous brood activity found in the present study most probably enhanced this effect additionally. Again, this seemed to be an effect of brood interruption, since recent studies did not find any direct effects of infestation levels on reproductive success of mites on colony level (Mondet et al. 2020b). Thus, reproductive success in single-infested cells seems to be altered by brood interruptions but not by the level of brood infestation itself. On the other hand, it is clear that the reproductive success of mites directly influences the mite population growth and is thus an important factor for the infestation level of brood cells (Nazzi and Le Conte 2015; Rosenkranz et al. 2010). Overall, the sharp contrast in infestation levels between colonies with brood interruption and those without points towards a general benefit of well-timed brood breaks for colony health.

5. CONCLUSION

The interruption of brood rearing clearly alters the reproductive success of mites, the recapping frequency and the brood infestation on colony level. It is therefore not only important for the survival of honeybee colonies, but may also interfere with measurements of resistance parameters.

Our results show for the first time that inhibiting the honeybee queen from egg laying for durations which are comparable to naturally occurring brood breaks can significantly reduce the probability for mite reproduction. In this case, brood interruptions mainly affected the proportion of infertile mother mites. This applies not only for the time of caging, but also for following brood cycles. After the brood interruption, however, the mite population seems to recover over time and regains normal reproductive abilities. How long this recovery takes seems to depend on the duration of the former brood interruption.

Reproductive failure of mites is one of the most accounted traits in honeybee science and breeding for resistance against *Varroa destructor*. The present study underlines the complexity of this trait as well as the challenges in comparable measurements of mite reproduction.

Despite the importance for standardized data acquisition, the lower reproductive success as well as the decreased mite infestation on colony level once again point to the beneficial aspects of summer brood interruptions in practical beekeeping.

SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at https://doi.org/10.1007/s13592-023-00998-x.

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AUTHOR CONTRIBUTION

All authors contributed to the elaboration of the study design. Sampling, statistical analysis and preparation of the first draft were performed by MG. In the following, RS, RB and MG commented on earlier versions of the manuscript and contributed in the writing process. The final manuscript version has been read and approved by all authors.

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DATA AVAILABILITY

The datasets generated and analysed during the present study are available from the corresponding author on reasonable request.

CODE AVAILABILITY

Not applicable.

DECLARATIONS

Ethics approval Not applicable.

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Conflict of interest The authors declare no competing interests.

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Supplementary materials:

Immediate and long-term effects of induced brood interruptions on the reproductive success of *Varroa destructor*

Examination of a subset of data gained during the first sampling (10 days after caging). At this time, queens of all treatment groups were restricted for the same duration. Thus, treatment groups were compared jointly with the unrestricted control group.

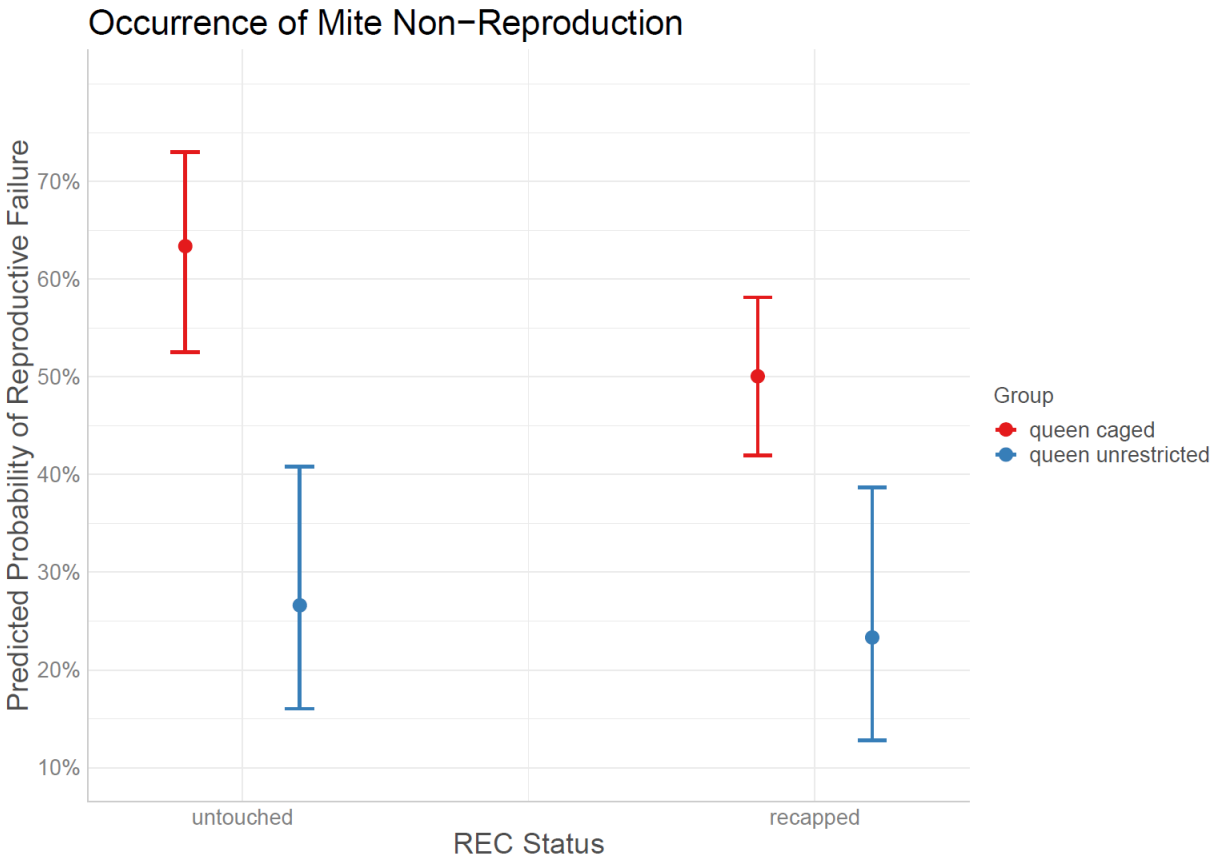

Figure I: Predicted probabilities of reproductive failure (MNR) of mother mites in brood combs sampled at day 10 after caging (displayed with 95% CI). Test statistics are given in the text.

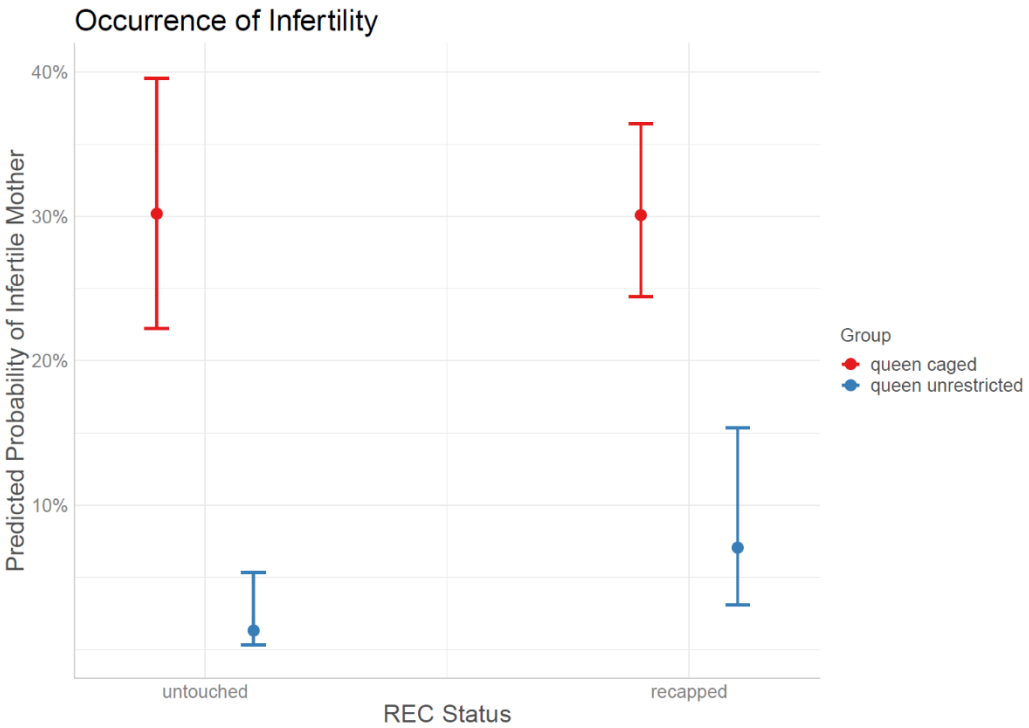

Figure II: Predicted probabilities of infertile mother mites in brood combs sampled at day 10 after caging (displayed with 95% CI). Test statistics are given in the text.

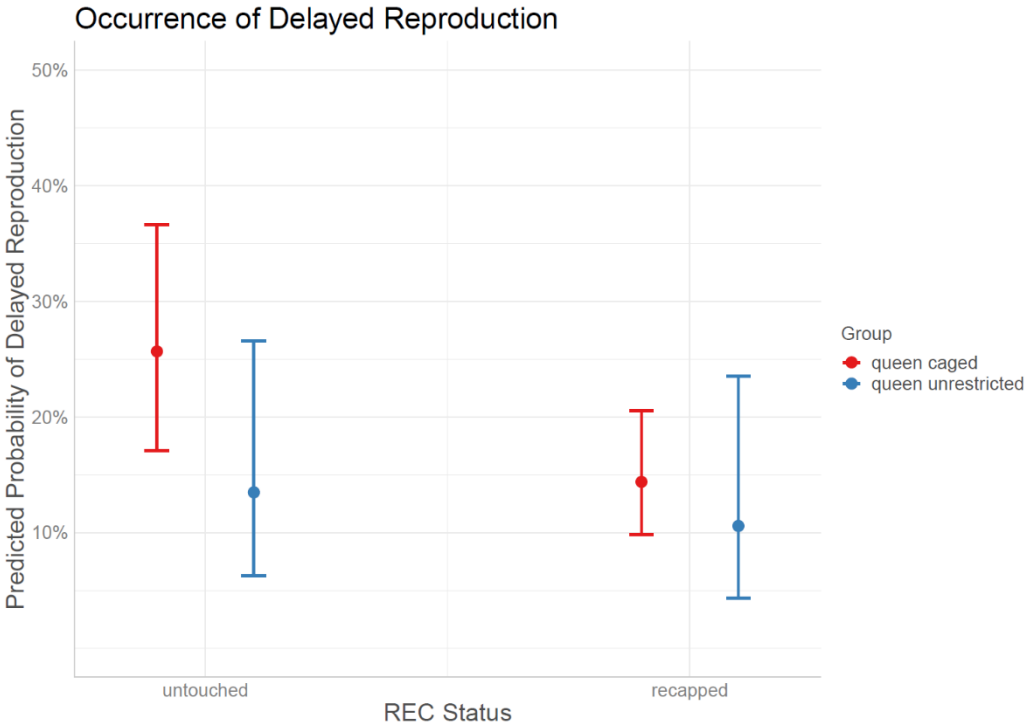

Figure III: Predicted probabilities of delayed reproduction in brood combs sampled at day 10 after caging (displayed with 95% CI). Test statistics are given in the text.

Figure IV: Predicted probabilities of missing males in brood combs sampled at day 10 after caging (displayed with 95% CI). Test statistics are given in the text.

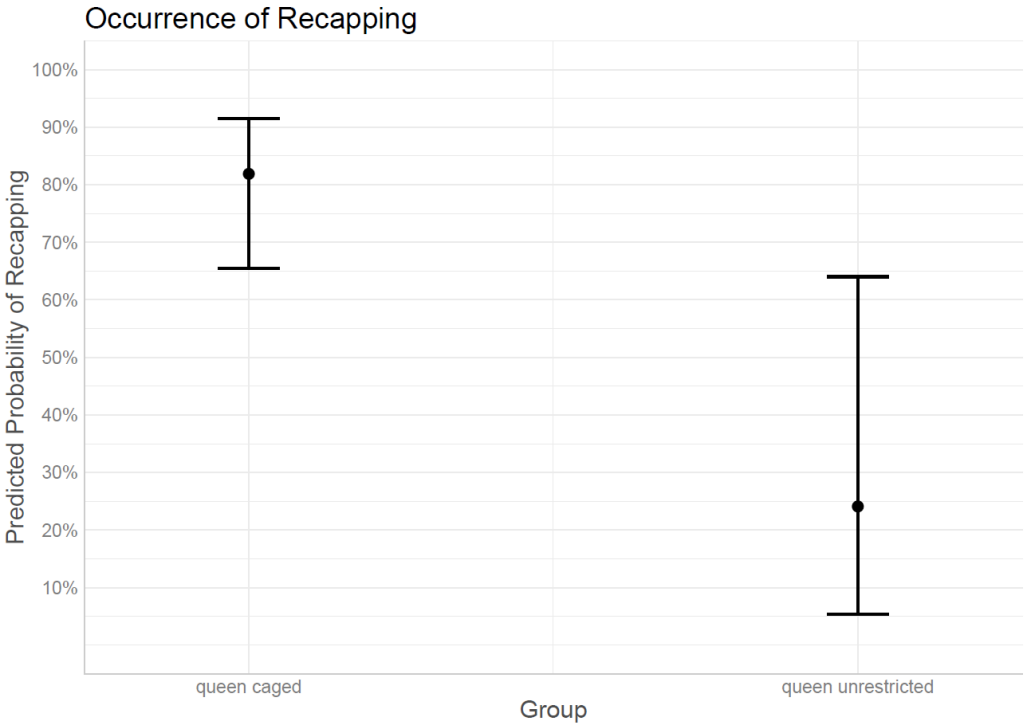

Figure V: Predicted probabilities of Recapping in brood combs sampled at day 10 after caging (displayed with 95% CI). Test statistics are given in the text.

Chapter III

Reproduction of *Varroa destructor* depends on well-timed host cell recapping and seasonal patterns

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Reproduction of *Varroa destructor* depends on well-timed host cell recapping and seasonal patterns

Martin Gabel[1,2]✉, Ricarda Scheiner[2], Ingolf Steffan-Dewenter[3] & Ralph Büchler[1]

Resistance traits of honeybees (*Apis mellifera*) against their major parasite *Varroa destructor* have fascinated scientists and breeders for long. Nevertheless, the mechanisms underlying resistance are still largely unknown. The same applies to possible interactions between host behaviours, mite reproduction and seasonal differences. Two resistance traits, reproductive failure of mites and recapping of brood cells, are of particular interest. High rates of recapping at the colony level were found to correspond with low reproductive success of mites. However, the direct effect of recapping on mite reproduction is still controversial and both traits seem to be very variable in their expression. Thus, a deeper knowledge of both, the effect of recapping on mite reproduction and the seasonal differences in the expression of these traits is urgently needed. To shed light on this host-parasite interaction, we investigated recapping and mite reproduction in full-grown colonies naturally infested with *V. destructor*. Measurements were repeated five times per year over the course of 3 years. The reproductive success of mites as well as the recapping frequency clearly followed seasonal patterns. Thereby, reproductive failure of mites at the cell level was constantly increased in case of recapping. Interestingly, this did not apply to the occurrence of infertile mites. In line with this, recapping activity in fertile cells was most frequent in brood ages in which mite offspring would be expected. Our results suggest that mite offspring is the main target of recapping. This, in turn, leads to a significantly reduced reproductive success of the parasite.

Resistance to *Varroa destructor* (hereafter referred to as *Varroa*) in honeybees is broadly described as the long-term survival of bee colonies without human treatment in a given habitat[1–3]. In this, the term comprises more detailed definitions of resistance (the host's ability to limit parasite burden) and tolerance (the host's ability to limit the harm caused) used for individual animals[4] and the ability to cope with various other environmental factors at the colony level. This became particularly evident when resistant honeybees were introduced into a foreign environment. There, their ability to overcome *Varroa* could no longer be observed[5–8]. The same applied to locally adapted mite-susceptible honeybees showing longer survival durations compared to foreign stock before they ultimately died from varroosis[5,8]. Resistance in honeybees therefore reflects a composition of various host-parasite interactions tuned to the respective environment[9,10], thereby increasing the duration of survival under the given conditions.

Various *Varroa*-resistance traits (i.e., traits that lower parasite burden) frequently co-occur in the same colony[10,11]. This displays a key feature of social immunity in honeybees[12] and fosters co-evolution from both sides of the host-parasite interaction[13,14]. Such host-parasite interactions form an equilibrium of bee and mite survival in several resistant honeybee populations[3,9,10]. However, the mechanisms behind this adaptation, i.e., the ultimate survival of colonies, can differ sharply[9,10]. Two distinct resistance traits have frequently been described as key mechanisms in surviving populations[3,9–12]: the uncapping and subsequent recapping of sealed brood cells (recapping, REC) and the reproductive failure of mites (mite non-reproduction, MNR[10], or suppressed mite reproduction, SMR sensu lato).

MNR describes any form of reproductive failure and thus comprises mother mites with either I) no offspring (infertile), II) only female offspring (missing male) or III) progeny which is too young to reach maturity before the host cell is expected to hatch (delayed reproduction)[15]. The different forms of MNR (infertile, no male or delayed), have been less intensively studied than MNR per se[16]. Yet, their contribution to the reproductive failure

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of *Varroa* (MNR) can differ considerably between populations[16] and thus likely reflects different background mechanisms.

REC was described to occur more frequently in naturally surviving colonies compared to susceptible ones[3,10,12], while low levels were even found in *Varroa-naïve* populations[17]. It thus seems to be a specific adaptation of basal brood hygiene behaviours to the parasite. However, the role of REC as stand-alone resistance trait or proxy for removal of infested brood cells (*Varroa*-sensitive hygiene, VSH) is still under debate[10,12,18,19]. If REC decreased the reproductive success of mites on its own, it could be much more cost-effective for the honeybee host than VSH, because no brood cells need to be sacrificed[12]. While this evolutionary cost saving seems to be obvious, the true benefit of REC as resistance trait for the colony appears to be largely unknown.

MNR and REC have thus gained increasing attention in studies on the biological basis of host-parasite interactions in honeybees. Their implementation as selection criteria in resistance breeding schemes[16,20], has led to a consensus on the need of a broader investigation of these traits[21].

The brood investigation required for this is tedious[15] and the accuracy of MNR and REC values strongly depends on sample size[22]. Since MNR seems to be the outcome of different background mechanisms[10], it shows a low phenotypic repeatability compared to REC and other resistance traits[22–24]. However, these changes might simply be linked to seasonal differences in the expression of underlying behaviours (e.g., VSH or REC) due to changing nectar flows[25], brood rearing activity[26] or other unknown factors. Up to now, such possible effects of seasonal variation on MNR remain largely unclear. The same applies to seasonal variation of REC and its effect on mite reproduction[10,12,18,19].

Since the set of resistance traits seems to be evolutionary tailored to the respective environment, their importance for the colony likely varies not only spatially but also temporally with external factors. The diversity of resistance traits found in naturally selected honeybee populations[9,10] thus might also display an adaptation to temporally changing conditions.

We investigated the reproductive success of *Varroa* and the occurrence of REC in 15 consecutive trials covering three beekeeping seasons (20, 20 and 15 colonies each) to shed light on possible seasonal variations in the behaviour of mites and bees.

We thereby directly linked REC at the brood cell level (> 4100 single-infested cells) to different forms of failure in mite reproduction to gain insight into the interaction of host and parasite. In addition to the measurements at the seasonal scale, we investigated the temporal occurrence of REC and brood termination (i.e., the lethal removal of brood by worker bees) during the capped brood stage. Therefore, nearly 116,000 age-defined cells were examined using a novel image-based approach.

Results

Reproductive success of mites is lower in recapped cells

The probability of MNR was significantly increased in recapped cells compared to untouched cells ($\chi^2 = 10.33$, df = 1, $p = 0.001$, Table 1). This general pattern was displayed on all sampling dates (Fig. 1a).

When investigating the underlying cause of reproductive failure, the occurrence of delayed reproduction was also significantly increased in recapped cells ($\chi^2 = 9.15$, df = 1, $p = 0.003$, Table 1, Fig. 1c). Likewise, male offspring was missing more often in recapped cells ($\chi^2 = 8.10$, df = 1, $p = 0.004$, Table 1, Fig. 1d).

Notably, the occurrence of infertile mites did not differ between recapped and untouched cells throughout all sampling points ($\chi^2 = 0.13$, df = 1, $p = 0.72$, Table 1, Fig. 1b).

Recapping frequency differs between reproductive states

The probability of REC differed significantly between brood cells with different reproductive states of *Varroa* mites ($\chi^2 = 18.03$, df = 3, $p < 0.001$, Table 1, Fig. 2). Recapping frequency was higher in non-reproductive cells (i.e., cells with infertile mothers, delayed reproduction or missing males, n = 1480; 45.2%) compared to reproductive cells (n = 2626; 40.78%) over all single-infested cells (n = 4106, $p < 0.001$, Table 2). This held true when cells with delayed reproduction (n = 629) or missing males (n = 213) were compared to reproductive cells respectively

Dependent	Parameter	n	df	X^2	*p*
Non-reproductive cells (MNR)	Recapping	4106 single-infested cells (45 colonies in 3 years)	1	10.33	0.001
	Sampling date		14	152.23	< 0.001
Infertile	Recapping		1	0.13	0.715
	Sampling date		14	94.7	< 0.001
Delayed	Recapping		1	9.15	0.003
	Sampling date		14	120.91	< 0.001
No male	Recapping		1	8.1	0.004
	Sampling date		14	32.22	0.004
Recapping	Sampling date		14	335.5	< 0.001
	Reproductive state		3	18.03	< 0.001

Table 1. Model output for factors affecting the reproductive success of mites and the recapping behaviour of bees. GLMMS of the binomial family were fitted to the data using the above-given parameters and dependent variables as well as colony and year as random factors.

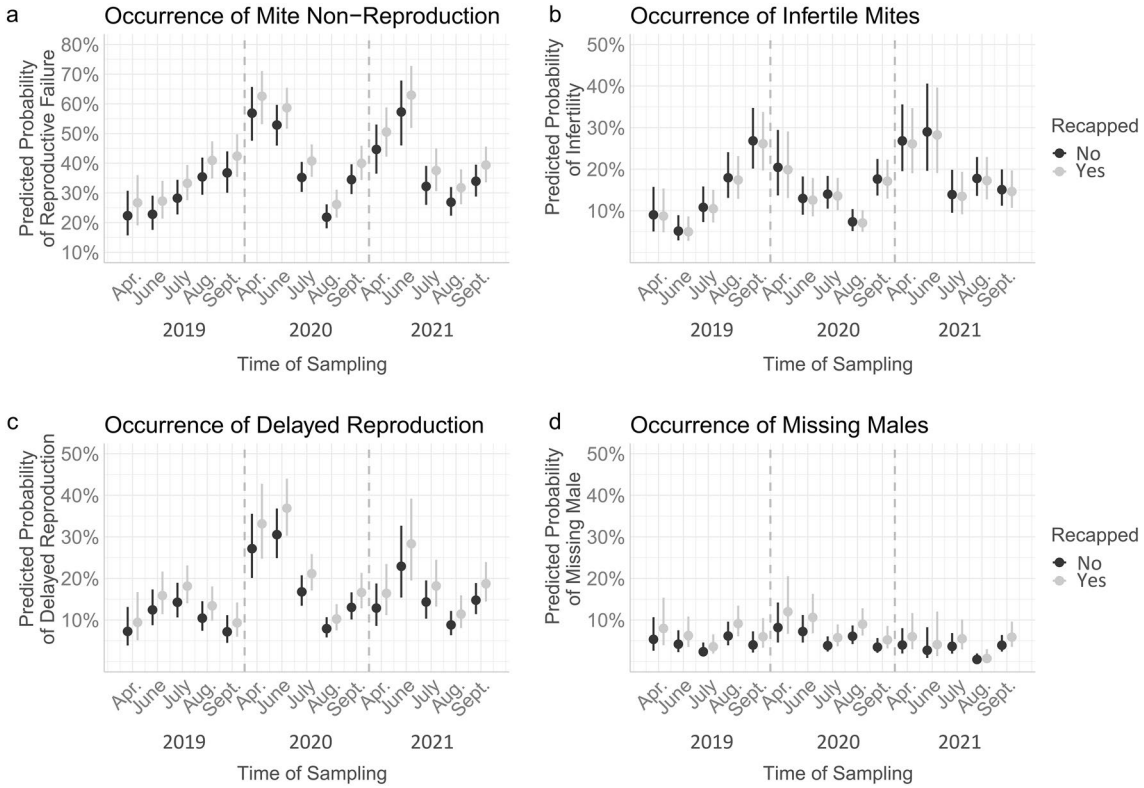

Figure 1. Predicted probabilities of (**a**) reproductive failure (MNR), as well as (**b**) infertility, (**c**) delayed reproduction and (**d**) missing males as cause for MNR (displayed with 95% CI). Vertical dashed lines separate consecutive years. Test statistics are given in Table 1, post-hoc comparisons are denoted in the supplementary material Table 4–7.

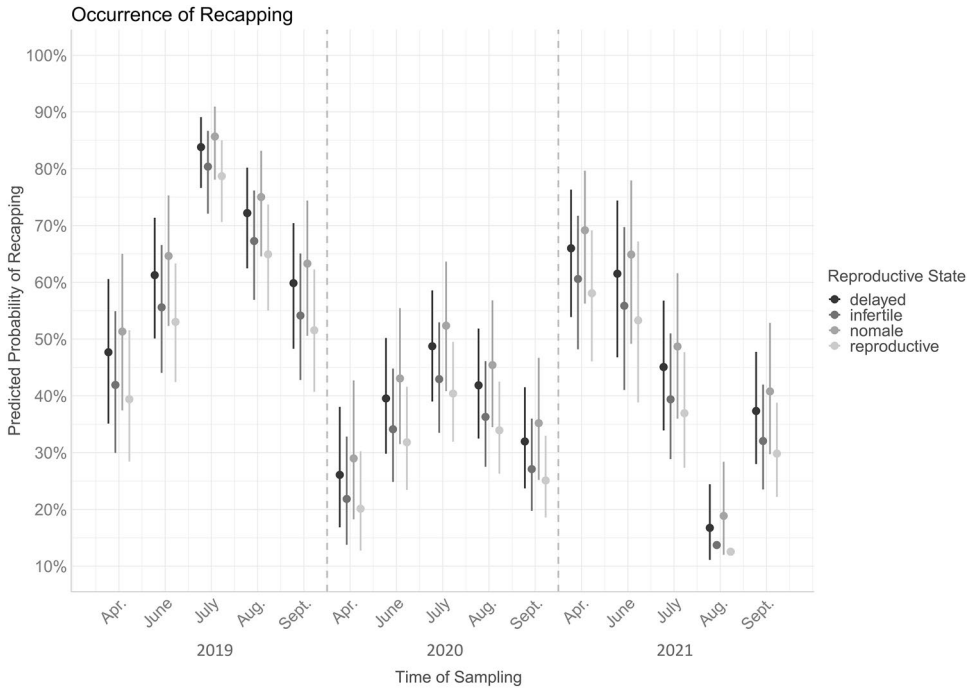

Figure 2. Predicted probabilities of recapping in single-infested cells (displayed with 95% CI). Vertical dashed lines separate consecutive years. Test statistics are given in Table 1, post-hoc comparisons are given in Table 2 and the supplementary material Table 8.

Comparison	Estimate	Z	p
Non-reproductive—reproductive	**−0.265**	**−3.510**	**<0.001**
Infertile—reproductive	0.104	1.000	0.75
Delayed—reproductive	**0.338**	**3.322**	**0.005**
No male—reproductive	**0.482**	**3.073**	**0.011**
Infertile—no male	−0.378	−2.133	0.143
Delayed—infertile	0.234	1.808	0.27
Delayed—no male	−0.144	−0.823	0.844

Table 2. Pairwise comparisons of recapping frequency in single-infested cells with different reproductive states. Factors denoted in bold indicate significant differences between groups ($p < 0.05$; Tukey-Method adjusted for comparing 4 estimates and averaged over sampling time in case of cause comparisons).

(47.54%, $p = 0.005$ and 53.52%; $p = 0.011$, Tab. 2). REC was observed in 40.13% of infertile cells (n = 638) which did not differ from the frequency in reproductive cells (Table 2). Among the non-reproductive cells, recapping frequency did not differ between the individual causes of failure (Table 2).

Mite reproduction follows seasonal patterns

The occurrence of MNR strongly differed between different sampling dates throughout the season ($\chi^2 = 152.23$, df = 14, $p < 0.001$, Table 1). While the probability of MNR increased steadily from April to September in 2019, it showed different patterns in 2020 and 2021 (Fig. 1a). In the latter years, failed reproduction was most frequently found between April and June, while it was least frequently observed at the end of August and beginning of September, respectively (Fig. 1a, supplementary material Table 4). This seasonal pattern was characterized by significantly higher probabilities of reproductive failure early in the season compared to mid-season brood cycles (Fig. 1a, supplementary material Table 4). The occurrence of each of the three causes for MNR was also significantly affected by the time of the season (Table 1, Fig. 1b–d).

Recapping frequency follows seasonal patterns

Overall, occurrence of REC differed significantly between sampling dates ($\chi^2 = 335.5$, df = 14, $p < 0.001$, Fig. 2, Table 1). In 2019 and 2020, infested cells tended to be recapped more frequently in mid-season, while in 2021 this occurred more frequently in spring (Fig. 2, supplementary material Table 8).

Colony level factors

MNR-Values and brood infestation showed a slightly negative correlation at the colony level ($r(136) = -0.19$, $p = 0.03$, Table 3). In turn, positive correlations were found between REC of all cells investigated (RECall) and brood infestation ($r(133) = 0.47$, $p < 0.01$, Table 3), as well as between RECall and bee infestation ($r(133) = 0.43$, $p < 0.01$, Table 3). No such correlation was found between RECinf (i.e., REC of infested cells) and any of the infestation measurements (Table 3).

There was a positive correlation between image-based REC values and RECinf ($r(133) = 0.27$, $p < 0.01$) and RECall ($r(131) = 0.49$, $p < 0.01$) values derived from classical brood analysis (Table 3). The same applied to image-based REC values and brood infestation ($r(134) = 0.35$, $p < 0.01$) and bee infestation ($r(134) = 0.31$, $p < 0.01$, Table 3). Brood termination rates were likewise correlated with RECinf ($r(133) = 0.2$, $p = 0.02$) and RECall ($r(131) = 0.44$, $p < 0.01$), as well as bee ($r(134) = 0.47$, $p < 0.01$) and brood infestation ($r(134) = 0.44$, $p < 0.01$, Table 3). Brood termination rates and image-based REC were also positively correlated ($r(134) = 0.33$, $p < 0.01$, Table 3).

	RECall	RECinf	Brood-infestation	Bee-infestation	Proportion of infertile mites	Termination rate	Image-based REC
MNR	$r(133) = -.05$, $p = .58$	$r(135) = .04$, $p = .61$	**$r(136) = -.19$, $p = .03$**	$r(136) = -.07$, $p = .39$	$r(136) = -.03$, $p = .71$	$r(134) = .00$, $p = .98$	$r(134) = -.01$, $p = .94$
RECall		**$r(133) = .74$, $p < .01$**	**$r(133) = .47$, $p < .01$**	**$r(133) = .43$, $p < .01$**	$r(133) = -.01$, $p = .89$	**$r(131) = .44$, $p < .01$**	**$r(131) = .49$, $p < .01$**
RECinf			$r(135) = -.06$, $p = .47$	$r(135) = -.07$, $p = .40$	**$r(136) = -.21$, $p = .01$**	**$r(133) = .02$, $p = .02$**	**$r(133) = .27$, $p < .01$**
Brood-infestation				**$r(135) = .47$, $p < .01$**	$r(135) = .06$, $p = .48$	**$r(134) = .44$, $p < .01$**	**$r(134) = .35$, $p < .01$**
Bee-infestation					$r(136) = .10$, $p = .26$	**$r(134) = .47$, $p < .01$**	**$r(134) = .31$, $p < .01$**
Proportion of infertile mites						$r(134) = .01$, $p = .89$	$r(134) = .03$, $p = .71$
Termination-rate							**$r(134) = .33$, $p < .01$**

Table 3. Correlations (Spearman) between colony level factors. Brood samples with less than 25 single-infested cells were excluded from calculations. Significant correlations are denoted in bold.

Frequency of recapping and cell termination differs between brood ages

In total, 115,943 age defined cells were investigated, of which 104,898 cells (90.47%) developed normally (i.e., were not terminated). Frequency of brood cell termination differed significantly between brood ages ($\chi^2 = 3783.6$, df = 4, $p < 0.001$). Distinctively more cells were found empty at day 10 post capping compared to younger brood stages ($p < 0.005$, each, Fig. 3b). Cells terminated after initial recapping were excluded from recapping analysis. Recapping was observed in 764 cells, of which 609 cells showed a single recapping event and 155 cells were recorded uncapped on two or more days. Only 28 multiply recapped cells were recorded sealed in between. For the remaining multiply recapped cells it is unclear whether they were sealed between pictures or remained uncapped ("bald brood") for longer periods. Recapping activity differed significantly between brood ages ($\chi^2 = 238.13$, df = 4, $p < 0.001$). Comparing all days, it was lowest at day two post capping ($p < 0.005$, each) and most frequently found six days post capping ($p < 0.001$, each, Fig. 3a).

Discussion

Our results clearly show that *Varroa* reproduction was significantly reduced in naturally recapped brood cells. Although REC was frequently described as an important resistance trait[3,10,11,27], beneficial effects for the host seem to be highly variable. At the colony level, high rates of REC were found to decrease *Varroa* reproduction in some cases[28,29], while this could not be confirmed in others[17,23]. At the cell level, the results were likewise variable: effects on MNR were mainly shown for artificially uncapped cells[12], while either no effect was found in naturally recapped cells[12,18,26] or results differed between sample sets[24]. Thus, it was proposed that the effect of REC may sometimes be overshadowed by other mechanisms[18,26]. This would also explain contradicting reports on the relationship between REC and infestation measures at the colony level[18,23,24,29,30]. Accordingly, we observed no correlation between RECinf and infestation measures or RECinf and MNR at the colony level (Table 3), although MNR was increased in the case of REC at the cell level (Fig. 1a, Table 1).

At the host colony level, however, beneficial effects have been indicated by a slight negative correlation between MNR and brood infestation (Table 3). This supports earlier reports on increased MNR values and lower infestation levels in surviving populations[1,3,10,27].

Thus, our findings support the formerly described diffuse effects of REC at the colony level but highlight its directly suppressing effects on mite reproduction at the cell level.

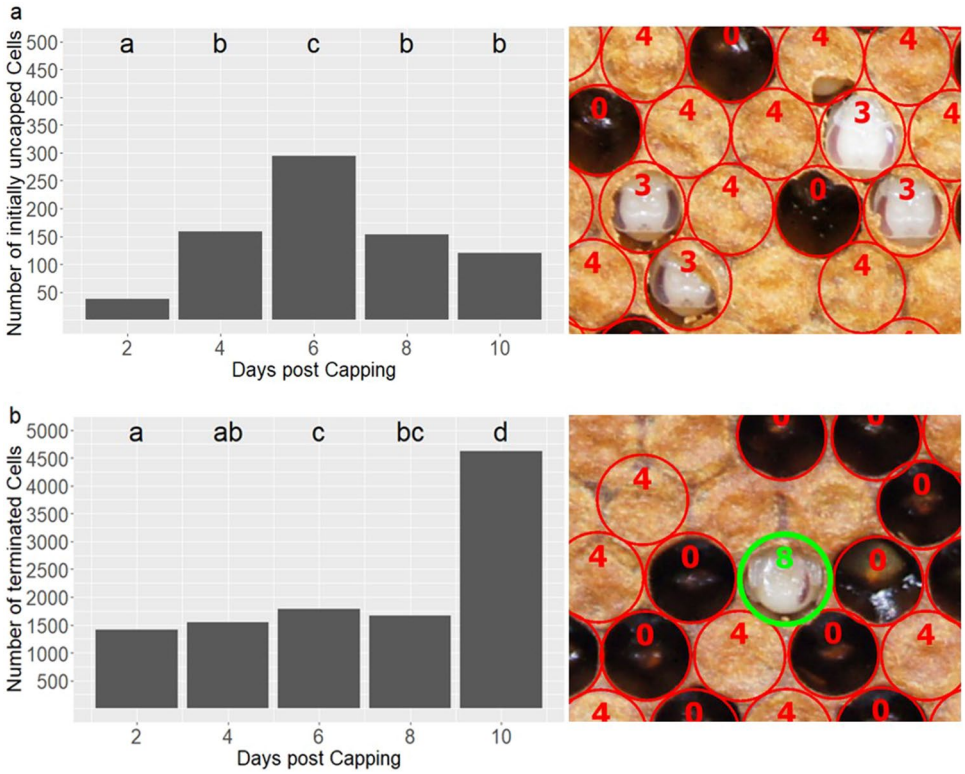

Figure 3. Time of (**a**) initial uncapping before REC and (**b**) cell termination with pictures taken during image-based brood investigation of (**a**) uncapped cells encoded with "3" and (**b**) terminated cell encoded with "8". The cell codes "0" and "4" refer to empty and sealed brood cells, respectively. Brood age (days post capping) had a significant effect on the time of initial uncapping (*GLMM:* $\chi^2 = 238.13$, df = 4, $p < 0.001$) and the time of brood termination (*GLMM:* $\chi^2 = 3783.6$, df = 4, $p < 0.001$). Different letters indicate significant differences between brood ages (Tukey-Method adjusted for comparing 5 estimates, $p < 0.05$ each).

REC holds the potential to disrupt different parts of the reproductive cycle of mites from the onset of egg laying to the mating of mature offspring[28]. By discriminating the different causes of reproductive failure, our results suggest that REC mainly affects fertile mites (i.e., mites with offspring). The proportions of missing males and delayed developing female offspring were significantly increased in recapped cells (Fig. 1a,d). This was also supported by results of the image-based brood analysis: Recapping mainly occurred after the first *Varroa* offspring should have hatched in fertile cells (Fig. 3a), i.e., four and six days post capping for male and female eggs, respectively[31–33]. This contradicts previous results in which the proportion of REC increased as pupal development progressed[17]. However, these findings were based on classical brood investigations and thus could not be adjusted for the accumulation of signs of REC (i.e., holes in the pupal cocoon) over time. In other words, older brood cells were per se more likely to show signs of REC, because bees had more time to express the behaviour. Thus, the time of initial REC cannot be reconstructed in classical brood investigations. The image-based investigation presented here reveals a more accurate impression of the timing of this host behaviour, which apparently depends on the ontogenesis of the parasite.

Such a targeting of fertile mites has been frequently discussed for REC[12] and the closely related behaviour VSH[17,34–36] but results appeared inconsistent among studies[19,37]. In the present study, the temporal link between the occurrence of uncapping and the suspected presence of mite offspring was less prominent in terminated cells than in recapped cells (Fig. 3a,b). However, cell termination may also be triggered by other causes, e.g., developmental abnormalities that mask such temporal patterns in *Varroa*-related brood termination. Notably, the sharp increase in empty cells 10 days post capping (Fig. 3b) was most probably an effect of faster development of some worker bees and the inaccuracy of approximately one day in the age definition method used. Thus, the timing of brood termination fits the timing of recapping and the ontogenesis of *Varroa* as discussed above.

Although it remains unclear which of the cells accounted by picture trials were actually infested by mites, cell termination rates correlated with VSH in earlier studies[38]. Termination rates in our dataset were correlated with bee and brood infestation as well as REC measurements at the colony level (Table 3), supporting these earlier findings[38]. Therefore in some cases, termination of initially uncapped cells may be a second step in a complex detection cascade leading to VSH as suggested before[17,39]. Nevertheless, cells being recapped instead of terminated after initial uncapping also showed significantly increased MNR values (Table 1). REC thus appears to work as a stand-alone resistance trait in other cases, underlining the complexity and redundancy of *Varroa*-resistance mechanisms.

In the latter case, our results point towards an effect of REC on the first two descendants, which are key players for successful reproduction. The first egg (male) is mostly laid in the forward cell section near the cap. Here it is better protected from the movements of the host larva[31]. This cell section, however, is especially exposed to disturbance by worker bees opening the cell lid (REC). Eggs laid near the cell lid are thus at risk to be removed by adult bees, as was recently shown for artificially inserted items[39]. Oviposition in the anterior part of the brood cell is also common for the second egg[31], which develops into the female with the best chances to reach maturity[33]. As[31] observed, these protonymphs are greatly challenged by crossing the legs of the host pupae towards the feeding side and are thus moving around "hyperactive" in the anterior cell section. Likewise to disturbance of sensitive eggs, bees opening the cell lid in this phase could thus also affect mobile protonymphs, e.g., because the mite offspring goes astray through the cell opening. Although following daughter mites do not face such in-cell-migration problems[31], a loss of the first daughter or the male would mostly be sufficient to prevent reproduction at the cell level because I) the remaining daughters would be too young to reach maturity in time (delayed reproduction) or II) adult daughters would miss a male for mating (no male). The loss of progeny would therefore explain the increased levels of delayed reproduction and missing males found after REC in this study (Fig. 1c,d). It also fits to earlier reports of decreased fecundity, i.e., the number of viable offspring in recapped cells[24].

In addition to the precision of targeted recapping[10,12,28], the exact timing thus seems to be crucial for the effectiveness of this resistance trait. This might also explain the results of[28], which found a lower number of daughter mites in colonies with enhanced REC. However, this pattern only held true in surviving colonies when mite-surviving and mite-susceptible colonies were analysed separately[28]. Therefore, REC seems to be beneficial in general but survivor populations might display a better timing of the behaviour which would reflect a key point of host-parasite-adaptation. We suggest further studies to focus on the exact timing of this resistance trait to unravel the effects of REC on fertile mites. In contrast to the commonly used brood investigation method[15], the detailed image-based approach of REC-measurements described here would better suit the needs of such studies. In turn, the standard method for MNR and REC measurements[15] is less laborious and thus seems to be more appropriate for large-scale investigations of breeding stocks and study populations.

In contrast to fertile mites, the occurrence of infertile mothers was not related to REC at the cell level (Table 1), although the respective proportions were slightly negative correlated at the colony level (Table 3). In line with this, the lowest REC rates were found in infertile cells (40.13%) compared to reproductive cells (40.78%) and non-reproductive cells caused by delayed reproduction (47.54%) or missing males (53.52%). This additionaly supports our assumption that fertile mites (i.e., mites with offspring) are the main target of REC activity as discussed above. At the same time, it seems to be uncommon for mother mites to invade uncapped cells since this would lead to increased infertility due to mismatching host brood signals[37,40]. Also, the previously supposed[24] mother mite emigration during uncapped brood periods seems to occur very rarely after natural infestation, since hardly any abandoned cells with mite faeces or orphan families were found. However, such emigration or removal events have been reported for cells artificially infested with mites deriving from the dispersal phase[39].

Over all, REC seems to affect mite offspring rather than mother mites in naturally infested cells.

Although independent of REC, the occurrence of mites without offspring (i.e., infertile mothers) strongly varied throughout the seasons rather than representing a stable base line (Fig. 1b). This supports the hypothesis that mite infertility is linked to other behaviours like selective VSH[19,34] which might in turn follow seasonal variations[25,26]. Such temporal effects are known for several resistance traits and the corresponding infestation

levels[25,26,41]. The expression of REC by the bee host and MNR by its parasite was likewise variable throughout our study period (Figs. 1, 2). This seasonal variation likely reflects a change in factors both inside and outside the colony: External factors such as changing nectar flows can alter resistance behaviours by shifting work force capacities[25]. The same applies to in-hive-factors like brood rearing[26,42] which again depend on the seasonality of the habitat. Changes in humidity and temperature could likewise have affected reproductive success, especially when combined with REC activity[43]. Notably, the pattern of reproductive success over the seasons 2020 and 2021 resemble earlier findings of[44,45], while the MNR expression in 2019 differed from this trend for unknown reasons. The MNR patterns in 2020 and 2021 might be explained by the changes of summer and winter bees[44,45], as well as brood breaks in winter time[26,42]. Thus, differences in brood rearing activity during the winter 2018/2019 and corresponding differences in worker longevity might also have led to the steady increase of MNR over the season 2019. However, neither the extend of brood rearing, nor the weather data was investigated in the present study and thus explanations for the differing seasonal patterns remain a subject of speculation. Nevertheless, the seasonal pattern reflects the dynamic character of host and parasite behaviours and underlines the challenges of comparable data acquisition. Although the expression of traits might often follow the patterns found in 2020 and 2021, as well as 1988 and 1989[44,45], the pattern of 2019 and the inter-season variation between months suggest that both temporal and spatial factors need to be accounted when comparing MNR and REC data of different colonies.

In practical bee breeding, this holds major importance for performance testing and targeted selection towards increased *Varroa*-resistance. The resistance traits MNR and REC were both found to be heritable and thus selectable, if the selection methods account for variability induced by outer effects[20]. The present results suggest that MNR and REC display valuable traits for resistance breeding although targeted selection might be greatly challenged by seasonal variation. This needs to be considered in performance testing and selection schemes, e.g., by using standardized methods and appropriate analyses of test data[20].

Our results prove that recapping behaviour of the host and mite reproduction are subject to considerable seasonal variation. Despite this overall variation at the seasonal level, the parasite's reproductive success was constantly decreased in recapped cells. In this, increased shares of delayed reproduction and missing males were linked to REC at the cell level. REC thus holds the potential as a stand-alone resistance trait but seems to add up to other mechanisms causing infertility and overall seasonal variation.

MNR and REC therefore appear to be valuable candidate traits for targeted selection towards increased *Varroa*-resistance. However, their temporal variation and other external factors need to be considered whenever measuring the expression of these traits.

Methods

Experimental setup

The study was conducted between 2019 and 2021 at the Bee Institute Kirchhain (Landesbetrieb Landwirtschaft Hessen, Hesse, Germany). The full-grown colonies derived from the Institute's Carniolan breeding stock. In 2019 and 2020, 20 colonies were investigated, while 15 colonies were examined in 2021. All samples were gained at the same apiary. Colonies were uniformly re-queened with young queens after the last sampling of the respective season. At the same time, oxalic acid was applied as late summer treatment against *Varroa*. Except of the sampling of brood and bees, no *Varroa*-treatments or swarm prevention measures were applied during the study season. All hives were managed uniformly according to the local beekeeping practice but did not receive winter treatments against *Varroa*.

Data collection

Colonies were sampled five times over the course of each beekeeping season (i.e., annually from April to September) at approximately monthly intervals as follows.

Picture trials and sampling of brood combs

One comb with predominantly L5 larvae was chosen per colony to obtain brood of similar age. The brood comb was marked, photographed from both sides and returned to the brood chamber. Afterwards, another six consecutive pictures were taken at two days intervals up to day 12 after the first picture (i.e., approximately 10 days after capping). Thus, the picture dates partly overlapped with the intervals given by[46] (see supplementary material Table 9). To ensure equal photo quality, the combs were mounted in a shaded box with a fixed distance of approximately 75 cm to the camera (Sony SLT-A33 with lens SAL1855, Sony Corp., Tokyo, Japan and Nikon D7500 with lens AF-S DX NIKKOR 18–300 mm, Nikon Corp., Tokyo, Japan). After the last picture of each trial (picture 7 of the respective comb, approximately 10 days after capping) brood combs were sampled and stored at − 20 °C until further brood investigation. Bee samples for standard infestation measurements were taken at the beginning of each picture trial[47]. Colonies with previous brood interruptions (e.g., due to swarming tendencies or queen change) were excluded from further analysis.

Brood investigation

The investigation of recapping and reproductive failure of mites was over all performed according to the RNSBB protocol[15]. Yet, for colony level factors, the minimum sample size per comb was reduced to 25 single-infested cells, due to low infestation levels early in the season. Brood combs were investigated using a stereo microscope (S9i, Leica Microsystems, Wetzlar, Germany) with ten-to-30-fold magnification. The reproduction of mites was classified depending on the respective brood age as either I) successful (i.e., normal amount and age of offspring), II) infertile (i.e., no offspring at all), III) no male (i.e., only female offspring of the right age) or IV) delayed (i.e., progeny too young to reach maturity before host cell hatch). Recapping at the colony level was calculated as the

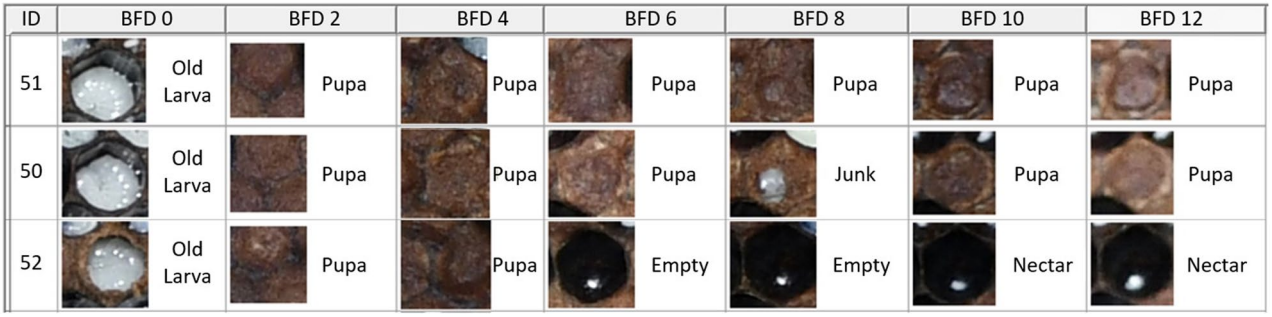

Figure 4. Picture trials of cell wise brood development starting approximately one day before capping (brood fixation day (BFD) 0, picture 1) up to approximately one day before emergence (BFD12, picture 7) in two days intervals (see supplementary material Table 9). Cell ID51: normal development, cell ID50: recapping at BFD8, cell ID52: brood termination at BFD6. Note that capped brood cells are always identified as "Pupa" although pupation is not completed at BFD 2 (picture 2). Uncapped brood cells are classified as "Junk" due to given category names predefined by the software.

proportion of recapped cells on the total number of investigated cells, i.e., infested and uninfested (RECall), and the number of infested cells only (RECinf).

Image-based brood investigation

Brood development was accounted cell wise based on the picture trials. Only age defined brood cells (L5 on picture 1 and sealed on picture 2) were used for further investigation (n = 115,943 cells). Alignment of pictures, cell determination and brood classification was performed using the software HiveAnalyzer (Version 2.33, Visionalytics, Pleidelsheim, Germany). All automatic steps of picture alignment and brood classification[48] were individually checked and manually corrected if needed. This especially applies for uncapped brood cells ("bald brood") which could not be identified automatically (Figs. 3a, 4). Uncapped cells which were sealed in a following picture were counted as recapped (Fig. 4). Brood cells which were uncapped and sealed several times were accounted in a separate category (multiply recapped). Cells which showed unusual development (i.e., any other cell content than sealed or uncapped brood after picture 2) were counted as terminated (Figs. 3b, 4).

Statistical analyses

The R environment (version 4.1.0, R Core Team 2021) was used for statistical analyses. Generalized linear mixed-effect models (glmer) from the binomial family (logit) were conducted to estimate the probabilities of recapping and different forms of non-reproduction at the cell level[49]. The occurrence of recapped cells and non-reproductive mites (including different types of failure) was considered as response variable. Time (i.e., date of sampling) and recapping status or reproductive state of the cell were implemented as fixed explanatory variables. Individual colonies were included as a random factor. The same applied for beekeeping seasons to account for sample clusters within each year. Day post capping was implemented as fixed variable for image-based recordings of the first recapping event and brood termination. In this case, colony and individual cell were used as random factors. The DHARMa package[50] was used to account for residuals and over-dispersion. Tukey-post-hoc tests (emmeans package[51]) were performed as subsequent pairwise comparisons among factor levels. For colony level measurements, spearman rank correlations were calculated using the psych package[52]. Samples with less than 25 single-infested cells were excluded from these analyses.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

All authors contributed to the elaboration of the study design. Sampling, statistical analysis and preparation of the first draft were performed by M.G. Afterwards, M.G., R.S., ISD and R.B. commented on subsequent versions of the manuscript. The final manuscript version was prepared by MG and has been read and approved by all authors.

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Competing interests

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Supplementary Information

Reproduction of *Varroa destructor* depends on well-timed host cell recapping and seasonal patterns

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Supplementary Information Table 4: Pairwise comparisons of the occurrence of MNR in single infested cells over the study period. Factors denoted in bold indicate significant differences between groups ($p < 0.05$; Tukey-Method adjusted for comparing 15 estimates and averaged over recapping status of cells).

Contrast	Estimate	Z	p
April 2019 - June 2019	-0.029	-0.109	1.000
April 2019 - July 2019	-0.314	-1.246	0.996
April 2019 - August 2019	-0.645	-2.593	0.375
April 2019 - September 2019	-0.705	-2.730	0.286
April 2019 - April 2020	**-1.524**	**-5.344**	**<0.001**
April 2019 - June 2020	**-1.362**	**-5.401**	**<0.001**

April 2019 - July 2020	-0.637	-2.679	0.318
April 2019 - August 2020	0.028	0.117	1.000
April 2019 - September 2020	-0.605	-2.520	0.426
April 2019 - May 2021	**-1.032**	**-3.780**	**0.013**
April 2019 - June 2021	**-1.540**	**-4.900**	**<0.001**
April 2019 - July 2021	-0.502	-1.907	0.850
April 2019 - August 2021	-0.246	-0.989	1.000
April 2019 - September 2021	-0.581	-2.370	0.538
June 2019 - July 2019	-0.285	-1.420	0.986
June 2019 - August 2019	-0.617	-3.118	0.112
June 2019 - September 2019	-0.676	-3.221	0.084
June 2019 - April 2020	**-1.495**	**-6.092**	**<0.001**
June 2019 - June 2020	**-1.333**	**-6.459**	**<0.001**
June 2019 - July 2020	-0.609	-3.258	0.075
June 2019 - August 2020	0.057	0.296	1.000

June 2019 - September 2020	-0.576	-3.040	0.138
June 2019 - May 2021	**-1.003**	**-4.345**	**0.001**
June 2019 - June 2021	**-1.511**	**-5.422**	**<0.001**
June 2019 - July 2021	-0.473	-2.153	0.698
June 2019 - August 2021	-0.218	-1.067	0.999
June 2019 - September 2021	-0.553	-2.775	0.260
July 2019 - August 2019	-0.332	-1.881	0.863
July 2019 - September 2019	-0.391	-2.057	0.764
July 2019 - April 2020	**-1.210**	**-5.256**	**<0.001**
July 2019 - June 2020	**-1.048**	**-5.518**	**<0.001**
July 2019 - July 2020	-0.324	-1.944	0.831
July 2019 - August 2020	0.342	1.978	0.812
July 2019 - September 2020	-0.291	-1.709	0.931
July 2019 - May 2021	-0.718	-3.335	0.060
July 2019 - June 2021	**-1.226**	**-4.603**	**<0.001**

July 2019 - July 2021	-0.188	-0.922	1.000
July 2019 - August 2021	0.067	0.357	1.000
July 2019 - September 2021	-0.268	-1.468	0.981
August 2019 - September 2019	-0.059	-0.324	1.000
August 2019 - April 2020	**-0.879**	**-3.867**	**0.009**
August 2019 - June 2020	**-0.716**	**-3.859**	**0.010**
August 2019 - July 2020	0.008	0.048	1.000
August 2019 - August 2020	**0.674**	**4.008**	**0.005**
August 2019 - September 2020	0.040	0.244	1.000
August 2019 - May 2021	-0.387	-1.824	0.889
August 2019 - June 2021	**-0.895**	**-3.395**	**0.049**
August 2019 - July 2021	0.143	0.716	1.000

August 2019 - August 2021	0.399	2.174	0.684
August 2019 - September 2021	0.064	0.360	1.000
September 2019 - April 2020	**-0.819**	**-3.458**	**0.040**
September 2019 - June 2020	-0.657	-3.339	0.059
September 2019 - July 2020	0.067	0.384	1.000
September 2019 - August 2020	**0.733**	**4.057**	**0.004**
September 2019 - September 2020	0.100	0.561	1.000
September 2019 - May 2021	-0.327	-1.473	0.980
September 2019 - June 2021	-0.835	-3.076	0.126
September 2019 - July 2021	0.203	0.963	1.000
September 2019 - August 2021	0.458	2.360	0.545
September 2019 -	0.123	0.652	1.000

September 2021			
April 2020 - June 2020	0.162	0.708	1.000
April 2020 - July 2020	**0.886**	**4.187**	**0.003**
April 2020 - August 2020	**1.552**	**7.189**	**6.87293555401425 e-11**
April 2020 - September 2020	**0.919**	**4.318**	**0.001**
April 2020 - May 2021	0.492	1.962	0.821
April 2020 - June 2021	-0.016	-0.055	1.000
April 2020 - July 2021	**1.022**	**4.266**	**0.002**
April 2020 - August 2021	**1.278**	**5.733**	**<0.001**
April 2020 - September 2021	**0.943**	**4.291**	**0.002**
June 2020 - July 2020	**0.724**	**4.387**	**0.001**
June 2020 - August 2020	**1.390**	**8.157**	**<0.001**
June 2020 - September 2020	**0.757**	**4.521**	**0.001**
June 2020 - May 2021	0.330	1.551	0.968
June 2020 - June 2021	-0.178	-0.676	1.000

June 2020 - July 2021	**0.860**	**4.299**	**0.002**
June 2020 - August 2021	**1.115**	**6.146**	**<0.001**
June 2020 - September 2021	**0.780**	**4.414**	**0.001**
July 2020 - August 2020	**0.666**	**4.528**	**0.001**
July 2020 - September 2020	0.033	0.228	1.000
July 2020 - May 2021	-0.394	-2.022	0.786
July 2020 - June 2021	**-0.903**	**-3.606**	**0.025**
July 2020 - July 2021	0.136	0.748	1.000
July 2020 - August 2021	0.391	2.428	0.494
July 2020 - September 2021	0.056	0.361	1.000
August 2020 - September 2020	**-0.633**	**-4.233**	**0.002**
August 2020 - May 2021	**-1.060**	**-5.303**	**<0.001**
August 2020 - June 2021	**-1.568**	**-6.175**	**<0.001**

August 2020 - July 2021	-0.530	-2.849	0.221
August 2020 - August 2021	-0.275	-1.653	0.947
August 2020 - September 2021	**-0.610**	**-3.789**	**0.013**
September 2020 - May 2021	-0.427	-2.159	0.695
September 2020 - June 2021	**-0.935**	**-3.708**	**0.017**
September 2020 - July 2021	0.103	0.561	1.000
September 2020 - August 2021	0.359	2.207	0.660
September 2020 - September 2021	0.024	0.149	1.000
May 2021 - June 2021	-0.508	-1.879	0.864
May 2021 - July 2021	0.530	2.494	0.445
May 2021 - August 2021	**0.785**	**3.955**	**0.007**
May 2021 - September 2021	0.450	2.331	0.567

June 2021 - July 2021	**1.038**	**3.955**	**0.007**
June 2021 - August 2021	**1.294**	**5.148**	**<0.001**
June 2021 - September 2021	**0.959**	**3.880**	**0.009**
July 2021 - August 2021	0.256	1.390	0.988
July 2021 - September 2021	-0.079	-0.444	1.000
August 2021 - September 2021	-0.335	-2.111	0.728

Supplementary Information Table 5: Pairwise comparisons of the occurrence of infertile mother mites in single infested cells over the study period. Factors denoted in bold indicate significant differences between groups ($p < 0.05$; Tukey-Method adjusted for comparing 15 estimates and averaged over recapping status of cells).

Contrast	Estimate	Z	p
April 2019 - June 2019	0.611	1.453	0.982
April 2019 - July 2019	-0.204	-0.553	1.000
April 2019 - August 2019	-0.791	-2.255	0.624
April 2019 - September 2019	**-1.308**	**-3.705**	**0.017**
April 2019 - April 2020	-0.953	-2.427	0.494
April 2019 - June 2020	-0.408	-1.129	0.999

April 2019 - July 2020	-0.495	-1.450	0.983
April 2019 - August 2020	0.225	0.630	1.000
April 2019 - September 2020	-0.770	-2.269	0.614
April 2019 - May 2021	**-1.307**	**-3.523**	**0.033**
April 2019 - June 2021	**-1.415**	**-3.505**	**0.035**
April 2019 - July 2021	-0.488	-1.295	0.994
April 2019 - August 2021	-0.780	-2.232	0.641
April 2019 - September 2021	-0.583	-1.654	0.947
June 2019 - July 2019	-0.814	-2.345	0.556
June 2019 - August 2019	**-1.402**	**-4.235**	**0.002**
June 2019 - September 2019	**-1.919**	**-5.750**	**<0.001**
June 2019 - April 2020	**-1.564**	**-4.120**	**0.003**
June 2019 - June 2020	-1.018	-2.944	0.176
June 2019 - July 2020	**-1.106**	**-3.419**	**0.046**
June 2019 - August 2020	-0.385	-1.131	0.999

June 2019 - September 2020	**-1.380**	**-4.299**	**0.002**
June 2019 - May 2021	**-1.918**	**-5.389**	**<0.001**
June 2019 - June 2021	**-2.026**	**-5.196**	**<0.001**
June 2019 - July 2021	-1.099	-3.028	0.142
June 2019 - August 2021	**-1.391**	**-4.139**	**0.003**
June 2019 - September 2021	**-1.194**	**-3.524**	**0.032**
July 2019 - August 2019	-0.587	-2.338	0.561
July 2019 - September 2019	**-1.104**	**-4.345**	**0.001**
July 2019 - April 2020	-0.749	-2.392	0.521
July 2019 - June 2020	-0.204	-0.742	1.000
July 2019 - July 2020	-0.292	-1.198	0.997
July 2019 - August 2020	0.429	1.607	0.958
July 2019 - September 2020	-0.566	-2.342	0.559
July 2019 - May 2021	**-1.103**	**-3.869**	**0.009**
July 2019 - June 2021	**-1.212**	**-3.703**	**0.017**

July 2019 - July 2021	-0.285	-0.965	1.000
July 2019 - August 2021	-0.577	-2.190	0.672
July 2019 - September 2021	-0.380	-1.429	0.985
August 2019 - September 2019	-0.517	-2.348	0.554
August 2019 - April 2020	-0.162	-0.553	1.000
August 2019 - June 2020	0.383	1.527	0.972
August 2019 - July 2020	0.296	1.367	0.990
August 2019 - August 2020	**1.016**	**4.204**	**0.002**
August 2019 - September 2020	0.021	0.101	1.000
August 2019 - May 2021	-0.516	-1.961	0.821
August 2019 - June 2021	-0.624	-2.026	0.783
August 2019 - July 2021	0.303	1.110	0.999

August 2019 - August 2021	0.011	0.045	1.000
August 2019 - September 2021	0.208	0.863	1.000
September 2019 - April 2020	0.355	1.207	0.997
September 2019 - June 2020	**0.900**	**3.563**	**0.028**
September 2019 - July 2020	**0.813**	**3.720**	**0.016**
September 2019 - August 2020	**1.533**	**6.300**	**<0.001**
September 2019 - September 2020	0.539	2.504	0.437
September 2019 - May 2021	0.001	0.004	1.000
September 2019 - June 2021	-0.107	-0.346	1.000
September 2019 - July 2021	0.820	2.991	0.157
September 2019 - August 2021	0.528	2.221	0.650
September 2019 -	0.725	2.998	0.154

September 2021			
April 2020 - June 2020	0.545	1.812	0.894
April 2020 - July 2020	0.458	1.669	0.943
April 2020 - August 2020	**1.178**	**4.012**	**0.005**
April 2020 - September 2020	0.184	0.681	1.000
April 2020 - May 2021	-0.354	-1.143	0.998
April 2020 - June 2021	-0.462	-1.321	0.993
April 2020 - July 2021	0.465	1.472	0.980
April 2020 - August 2021	0.173	0.615	1.000
April 2020 - September 2021	0.370	1.292	0.994
June 2020 - July 2020	-0.088	-0.381	1.000
June 2020 - August 2020	0.633	2.500	0.440
June 2020 - September 2020	-0.362	-1.604	0.958
June 2020 - May 2021	-0.899	-3.294	0.068
June 2020 - June 2021	-1.008	-3.176	0.095

June 2020 - July 2021	-0.080	-0.286	1.000
June 2020 - August 2021	-0.372	-1.521	0.973
June 2020 - September 2021	-0.176	-0.703	1.000
July 2020 - August 2020	0.721	3.252	0.077
July 2020 - September 2020	-0.274	-1.452	0.982
July 2020 - May 2021	-0.812	-3.324	0.062
July 2020 - June 2021	-0.920	-3.139	0.106
July 2020 - July 2021	0.007	0.029	1.000
July 2020 - August 2021	-0.285	-1.338	0.992
July 2020 - September 2021	-0.088	-0.404	1.000
August 2020 - September 2020	**-0.995**	**-4.590**	**<0.001**
August 2020 - May 2021	**-1.532**	**-5.741**	**<0.001**
August 2020 - June 2021	**-1.640**	**-5.257**	**<0.001**

August 2020 - July 2021	-0.713	-2.595	0.373
August 2020 - August 2021	**-1.005**	**-4.240**	**0.002**
August 2020 - September 2021	-0.808	-3.341	0.058
September 2020 - May 2021	-0.537	-2.224	0.647
September 2020 - June 2021	-0.646	-2.221	0.650
September 2020 - July 2021	0.281	1.126	0.999
September 2020 - August 2021	-0.011	-0.052	1.000
September 2020 - September 2021	0.186	0.875	1.000
May 2021 - June 2021	-0.108	-0.371	1.000
May 2021 - July 2021	0.819	3.142	0.105
May 2021 - August 2021	0.527	2.316	0.578
May 2021 - September 2021	0.724	3.138	0.106

June 2021 - July 2021	0.927	3.053	0.134
June 2021 - August 2021	0.635	2.306	0.586
June 2021 - September 2021	0.832	2.996	0.155
July 2021 - August 2021	-0.292	-1.237	0.996
July 2021 - September 2021	-0.095	-0.397	1.000
August 2021 - September 2021	0.197	0.998	1.000

Supplementary Information Table 6: Pairwise comparisons of the occurrence of delayed reproduction in single infested cells over the study period. Factors denoted in bold indicate significant differences between groups ($p < 0.05$; Tukey-Method adjusted for comparing 15 estimates and averaged over recapping status of cells).

Contrast	Estimate	Z	p
April 2019 - June 2019	-0.597	-1.565	0.966
April 2019 - July 2019	-0.757	-2.062	0.760
April 2019 - August 2019	-0.400	-1.060	0.999
April 2019 - September 2019	0.013	0.031	1.000
April 2019 - April 2020	**-1.562**	**-4.043**	**0.005**
April 2019 - June 2020	**-1.727**	**-4.808**	**<0.001**

April 2019 - July 2020	-0.948	-2.678	0.319
April 2019 - August 2020	-0.094	-0.253	1.000
April 2019 - September 2020	-0.651	-1.802	0.898
April 2019 - May 2021	-0.635	-1.586	0.962
April 2019 - June 2021	-1.336	-3.238	0.080
April 2019 - July 2021	-0.760	-2.002	0.798
April 2019 - August 2021	-0.214	-0.565	1.000
April 2019 - September 2021	-0.796	-2.198	0.666
June 2019 - July 2019	-0.160	-0.654	1.000
June 2019 - August 2019	0.197	0.762	1.000
June 2019 - September 2019	0.610	1.990	0.805
June 2019 - April 2020	**-0.965**	**-3.496**	**0.036**
June 2019 - June 2020	**-1.130**	**-4.789**	**<0.001**
June 2019 - July 2020	-0.351	-1.545	0.970
June 2019 - August 2020	0.503	2.007	0.795

June 2019 - September 2020	-0.054	-0.226	1.000
June 2019 - May 2021	-0.038	-0.128	1.000
June 2019 - June 2021	-0.739	-2.379	0.531
June 2019 - July 2021	-0.163	-0.613	1.000
June 2019 - August 2021	0.383	1.435	0.984
June 2019 - September 2021	-0.199	-0.824	1.000
July 2019 - August 2019	0.357	1.526	0.973
July 2019 - September 2019	0.769	2.690	0.311
July 2019 - April 2020	-0.806	-3.151	0.102
July 2019 - June 2020	**-0.970**	**-4.591**	**<0.001**
July 2019 - July 2020	-0.191	-0.955	1.000
July 2019 - August 2020	0.663	2.918	0.188
July 2019 - September 2020	0.106	0.493	1.000
July 2019 - May 2021	0.122	0.445	1.000
July 2019 - June 2021	-0.580	-1.988	0.806

July 2019 - July 2021	-0.004	-0.016	1.000
July 2019 - August 2021	0.542	2.196	0.668
July 2019 - September 2021	-0.040	-0.182	1.000
August 2019 - September 2019	0.412	1.382	0.989
August 2019 - April 2020	**-1.163**	**-4.312**	**0.002**
August 2019 - June 2020	**-1.327**	**-5.819**	**<0.001**
August 2019 - July 2020	-0.548	-2.513	0.431
August 2019 - August 2020	0.306	1.259	0.996
August 2019 - September 2020	-0.251	-1.088	0.999
August 2019 - May 2021	-0.235	-0.818	1.000
August 2019 - June 2021	-0.937	-3.080	0.124
August 2019 - July 2021	-0.361	-1.389	0.988

August 2019 - August 2021	0.185	0.710	1.000
August 2019 - September 2021	-0.397	-1.685	0.938
September 2019 - April 2020	**-1.575**	**-5.020**	**<0.001**
September 2019 - June 2020	**-1.739**	**-6.235**	**<0.001**
September 2019 - July 2020	**-0.960**	**-3.540**	**0.031**
September 2019 - August 2020	-0.106	-0.364	1.000
September 2019 - September 2020	-0.664	-2.361	0.545
September 2019 - May 2021	-0.647	-1.965	0.819
September 2019 - June 2021	**-1.349**	**-3.916**	**0.008**
September 2019 - July 2021	-0.773	-2.533	0.416
September 2019 - August 2021	-0.227	-0.743	1.000
September 2019 -	-0.809	-2.851	0.220

September 2021			
April 2020 - June 2020	-0.164	-0.683	1.000
April 2020 - July 2020	0.615	2.637	0.345
April 2020 - August 2020	**1.469**	**5.751**	**<0.001**
April 2020 - September 2020	**0.911**	**3.760**	**0.014**
April 2020 - May 2021	0.928	3.104	0.117
April 2020 - June 2021	0.226	0.716	1.000
April 2020 - July 2021	0.802	2.967	0.167
April 2020 - August 2021	**1.348**	**5.051**	**<0.001**
April 2020 - September 2021	0.766	3.137	0.106
June 2020 - July 2020	**0.779**	**4.219**	**0.002**
June 2020 - August 2020	**1.633**	**7.690**	**<0.001**
June 2020 - September 2020	**1.076**	**5.461**	**<0.001**
June 2020 - May 2021	**1.092**	**4.153**	**0.003**
June 2020 - June 2021	0.390	1.385	0.989

June 2020 - July 2021	**0.966**	**4.192**	**0.003**
June 2020 - August 2021	**1.513**	**6.643**	**<0.001**
June 2020 - September 2021	**0.930**	**4.654**	**<0.001**
July 2020 - August 2020	**0.854**	**4.203**	**0.002**
July 2020 - September 2020	0.297	1.584	0.962
July 2020 - May 2021	0.313	1.226	0.997
July 2020 - June 2021	-0.388	-1.413	0.986
July 2020 - July 2021	0.187	0.844	1.000
July 2020 - August 2021	0.734	3.328	0.061
July 2020 - September 2021	0.151	0.794	1.000
August 2020 - September 2020	-0.557	-2.595	0.373
August 2020 - May 2021	-0.541	-1.959	0.823
August 2020 - June 2021	**-1.243**	**-4.219**	**0.002**

August 2020 - July 2021	-0.667	-2.714	0.296
August 2020 - August 2021	-0.121	-0.496	1.000
August 2020 - September 2021	-0.703	-3.235	0.080
September 2020 - May 2021	0.016	0.061	1.000
September 2020 - June 2021	-0.685	-2.413	0.505
September 2020 - July 2021	-0.110	-0.471	1.000
September 2020 - August 2021	0.437	1.904	0.852
September 2020 - September 2021	-0.145	-0.719	1.000
May 2021 - June 2021	-0.702	-2.131	0.714
May 2021 - July 2021	-0.126	-0.437	1.000
May 2021 - August 2021	0.421	1.466	0.981
May 2021 - September 2021	-0.162	-0.611	1.000

June 2021 - July 2021	0.576	1.889	0.859
June 2021 - August 2021	**1.122**	**3.686**	**0.019**
June 2021 - September 2021	0.540	1.905	0.851
July 2021 - August 2021	0.546	2.132	0.714
July 2021 - September 2021	-0.036	-0.155	1.000
August 2021 - September 2021	-0.582	-2.557	0.400

Supplementary Information Table 7: Pairwise comparisons of the occurrence of missing males in single infested cells over the study period. Factors denoted in bold indicate significant differences between groups ($p < 0.05$; Tukey-Method adjusted for comparing 15 estimates and averaged over recapping status of cells).

Contrast	Estimate	Z	p
April 2019 - June 2019	0.261	0.549	1.000
April 2019 - July 2019	0.849	1.720	0.927
April 2019 - August 2019	-0.151	-0.351	1.000
April 2019 - September 2019	0.305	0.638	1.000
April 2019 - April 2020	-0.456	-0.943	1.000
April 2019 - June 2020	-0.320	-0.733	1.000

April 2019 - July 2020	0.346	0.795	1.000
April 2019 - August 2020	-0.136	-0.329	1.000
April 2019 - September 2020	0.450	1.003	1.000
April 2019 - May 2021	0.307	0.586	1.000
April 2019 - June 2021	0.706	1.009	1.000
April 2019 - July 2021	0.397	0.798	1.000
April 2019 - August 2021	2.442	3.045	0.137
April 2019 - September 2021	0.323	0.719	1.000
June 2019 - July 2019	0.588	1.332	0.992
June 2019 - August 2019	-0.412	-1.110	0.999
June 2019 - September 2019	0.044	0.103	1.000
June 2019 - April 2020	-0.717	-1.642	0.950
June 2019 - June 2020	-0.581	-1.513	0.975
June 2019 - July 2020	0.085	0.223	1.000
June 2019 - August 2020	-0.397	-1.117	0.999

June 2019 - September 2020	0.189	0.476	1.000
June 2019 - May 2021	0.046	0.097	1.000
June 2019 - June 2021	0.445	0.669	1.000
June 2019 - July 2021	0.136	0.302	1.000
June 2019 - August 2021	2.181	2.814	0.239
June 2019 - September 2021	0.062	0.157	1.000
July 2019 - August 2019	-1.000	-2.585	0.380
July 2019 - September 2019	-0.544	-1.231	0.997
July 2019 - April 2020	-1.306	-2.873	0.209
July 2019 - June 2020	-1.169	-2.905	0.194
July 2019 - July 2020	-0.504	-1.267	0.995
July 2019 - August 2020	-0.985	-2.632	0.348
July 2019 - September 2020	-0.400	-0.963	1.000
July 2019 - May 2021	-0.542	-1.099	0.999
July 2019 - June 2021	-0.143	-0.211	1.000

July 2019 - July 2021	-0.453	-0.971	1.000
July 2019 - August 2021	1.593	2.025	0.783
July 2019 - September 2021	-0.526	-1.260	0.996
August 2019 - September 2019	0.456	1.251	0.996
August 2019 - April 2020	-0.306	-0.802	1.000
August 2019 - June 2020	-0.169	-0.534	1.000
August 2019 - July 2020	0.496	1.594	0.960
August 2019 - August 2020	0.015	0.053	1.000
August 2019 - September 2020	0.600	1.808	0.895
August 2019 - May 2021	0.458	1.065	0.999
August 2019 - June 2021	0.857	1.354	0.991
August 2019 - July 2021	0.547	1.382	0.989

August 2019 - August 2021	**2.593**	**3.475**	**0.038**
August 2019 - September 2021	0.474	1.406	0.987
September 2019 - April 2020	-0.762	-1.749	0.918
September 2019 - June 2020	-0.625	-1.643	0.949
September 2019 - July 2020	0.040	0.107	1.000
September 2019 - August 2020	-0.441	-1.259	0.996
September 2019 - September 2020	0.144	0.368	1.000
September 2019 - May 2021	0.002	0.004	1.000
September 2019 - June 2021	0.401	0.600	1.000
September 2019 - July 2021	0.091	0.204	1.000
September 2019 - August 2021	2.137	2.757	0.270
September 2019 -	0.018	0.045	1.000

September 2021			
April 2020 - June 2020	0.136	0.353	1.000
April 2020 - July 2020	0.802	2.096	0.738
April 2020 - August 2020	0.321	0.901	1.000
April 2020 - September 2020	0.906	2.286	0.601
April 2020 - May 2021	0.763	1.579	0.963
April 2020 - June 2021	1.163	1.733	0.923
April 2020 - July 2021	0.853	1.888	0.860
April 2020 - August 2021	**2.899**	**3.747**	**0.015**
April 2020 - September 2021	0.779	1.957	0.824
June 2020 - July 2020	0.666	2.072	0.754
June 2020 - August 2020	0.184	0.636	1.000
June 2020 - September 2020	0.770	2.271	0.613
June 2020 - May 2021	0.627	1.432	0.985
June 2020 - June 2021	1.026	1.607	0.958

June 2020 - July 2021	0.717	1.778	0.907
June 2020 - August 2021	**2.762**	**3.695**	**0.018**
June 2020 - September 2021	0.643	1.873	0.867
July 2020 - August 2020	-0.481	-1.689	0.937
July 2020 - September 2020	0.104	0.311	1.000
July 2020 - May 2021	-0.038	-0.088	1.000
July 2020 - June 2021	0.361	0.566	1.000
July 2020 - July 2021	0.051	0.128	1.000
July 2020 - August 2021	2.097	2.809	0.241
July 2020 - September 2021	-0.022	-0.066	1.000
August 2020 - September 2020	0.585	1.922	0.842
August 2020 - May 2021	0.443	1.073	0.999
August 2020 - June 2021	0.842	1.354	0.991

August 2020 - July 2021	0.532	1.421	0.986
August 2020 - August 2021	**2.578**	**3.519**	**0.033**
August 2020 - September 2021	0.459	1.481	0.979
September 2020 - May 2021	-0.143	-0.318	1.000
September 2020 - June 2021	0.257	0.397	1.000
September 2020 - July 2021	-0.053	-0.128	1.000
September 2020 - August 2021	1.993	2.647	0.338
September 2020 - September 2021	-0.127	-0.356	1.000
May 2021 - June 2021	0.399	0.576	1.000
May 2021 - July 2021	0.089	0.183	1.000
May 2021 - August 2021	2.135	2.671	0.323
May 2021 - September 2021	0.016	0.036	1.000

June 2021 - July 2021	-0.310	-0.459	1.000
June 2021 - August 2021	1.736	1.878	0.865
June 2021 - September 2021	-0.383	-0.597	1.000
July 2021 - August 2021	2.046	2.621	0.355
July 2021 - September 2021	-0.074	-0.179	1.000
August 2021 - September 2021	-2.119	-2.822	0.235

Supplementary Information Table 8: Pairwise comparisons of the occurrence of REC in single infested cells over the study period. Factors denoted in bold indicate significant differences between groups ($p < 0.05$; Tukey-Method adjusted for comparing 15 estimates and averaged over reproductive status of cells).

Contrast	Estimate	Z	p
April 2019 - June 2019	-0.549	-2.299	0.591
April 2019 - July 2019	**-1.735**	**-7.048**	**<0.001**
April 2019 - August 2019	-1.045	-4.363	0.001
April 2019 - September 2019	-0.490	-1.948	0.829
April 2019 - April 2020	0.950	3.104	0.116
April 2019 - June 2020	0.333	1.364	0.990

April 2019 - July 2020	-0.040	-0.179	1.000
April 2019 - August 2020	0.238	1.071	0.999
April 2019 - September 2020	0.665	2.874	0.208
April 2019 - May 2021	-0.754	-2.611	0.362
April 2019 - June 2021	-0.559	-1.666	0.943
April 2019 - July 2021	0.106	0.394	1.000
April 2019 - August 2021	**1.513**	**5.636**	**<0.001**
April 2019 - September 2021	0.427	1.714	0.929
June 2019 - July 2019	**-1.186**	**-5.782**	**<0.001**
June 2019 - August 2019	-0.496	-2.502	0.439
June 2019 - September 2019	0.059	0.275	1.000
June 2019 - April 2020	**1.499**	**5.332**	**<0.001**
June 2019 - June 2020	0.883	4.205	0.002
June 2019 - July 2020	0.509	2.760	0.269
June 2019 - August 2020	0.788	4.300	0.002

June 2019 - September 2020	**1.214**	**6.330**	**<0.001**
June 2019 - May 2021	-0.205	-0.781	1.000
June 2019 - June 2021	-0.010	-0.030	1.000
June 2019 - July 2021	0.656	2.713	0.297
June 2019 - August 2021	**2.062**	**8.530**	**<0.001**
June 2019 - September 2021	**0.976**	**4.453**	**0.001**
July 2019 - August 2019	**0.690**	**3.453**	**0.041**
July 2019 - September 2019	**1.245**	**5.799**	**<0.001**
July 2019 - April 2020	**2.685**	**9.527**	**<0.001**
July 2019 - June 2020	**2.068**	**9.761**	**<0.001**
July 2019 - July 2020	**1.695**	**9.119**	**<0.001**
July 2019 - August 2020	**1.973**	**10.665**	**<0.001**
July 2019 - September 2020	**2.400**	**12.408**	**<0.001**
July 2019 - May 2021	**0.981**	**3.732**	**0.016**
July 2019 - June 2021	**1.176**	**3.753**	**0.015**

July 2019 - July 2021	**1.841**	**7.562**	**<0.001**
July 2019 - August 2021	**3.248**	**13.323**	**<0.001**
July 2019 - September 2021	**2.162**	**9.771**	**<0.001**
August 2019 - September 2019	0.554	2.773	0.261
August 2019 - April 2020	**1.994**	**7.228**	**<0.001**
August 2019 - June 2020	**1.378**	**6.780**	**<0.001**
August 2019 - July 2020	**1.005**	**5.733**	**<0.001**
August 2019 - August 2020	**1.283**	**7.360**	**<0.001**
August 2019 - September 2020	**1.710**	**9.320**	**<0.001**
August 2019 - May 2021	0.291	1.133	0.999
August 2019 - June 2021	0.486	1.576	0.964
August 2019 - July 2021	**1.151**	**4.864**	**<0.001**

August 2019 - August 2021	**2.558**	**10.792**	**<0.001**
August 2019 - September 2021	**1.472**	**6.880**	**<0.001**
September 2019 - April 2020	**1.440**	**5.062**	**<0.001**
September 2019 - June 2020	**0.824**	**3.819**	**0.011**
September 2019 - July 2020	0.450	2.374	0.534
September 2019 - August 2020	**0.729**	**3.865**	**0.010**
September 2019 - September 2020	**1.155**	**5.874**	**<0.001**
September 2019 - May 2021	-0.264	-0.988	1.000
September 2019 - June 2021	-0.068	-0.216	1.000
September 2019 - July 2021	0.597	2.413	0.505
September 2019 - August 2021	**2.003**	**8.101**	**<0.001**
September 2019 -	**0.917**	**4.066**	**0.004**

September 2021			
April 2020 - June 2020	-0.616	-2.266	0.616
April 2020 - July 2020	**-0.990**	**-3.907**	**0.008**
April 2020 - August 2020	-0.711	-2.808	0.242
April 2020 - September 2020	-0.285	-1.098	0.999
April 2020 - May 2021	**-1.704**	**-5.511**	**<0.001**
April 2020 - June 2021	**-1.508**	**-4.277**	**0.002**
April 2020 - July 2021	-0.843	-2.895	0.198
April 2020 - August 2021	0.563	1.937	0.834
April 2020 - September 2021	-0.523	-1.923	0.842
June 2020 - July 2020	-0.374	-2.139	0.709
June 2020 - August 2020	-0.095	-0.543	1.000
June 2020 - September 2020	0.332	1.808	0.896
June 2020 - May 2021	**-1.087**	**-4.326**	**0.001**
June 2020 - June 2021	-0.892	-2.937	0.179

June 2020 - July 2021	-0.227	-0.984	1.000
June 2020 - August 2021	**1.179**	**5.087**	**<0.001**
June 2020 - September 2021	0.093	0.450	1.000
July 2020 - August 2020	0.279	1.968	0.817
July 2020 - September 2020	**0.705**	**4.654**	**<0.001**
July 2020 - May 2021	-0.714	-3.086	0.122
July 2020 - June 2021	-0.519	-1.801	0.898
July 2020 - July 2021	0.147	0.705	1.000
July 2020 - August 2021	**1.553**	**7.435**	**<0.001**
July 2020 - September 2021	0.467	2.566	0.393
August 2020 - September 2020	0.427	2.822	0.235
August 2020 - May 2021	**-0.992**	**-4.293**	**0.002**
August 2020 - June 2021	-0.797	-2.765	0.266

August 2020 - July 2021	-0.132	-0.638	1.000
August 2020 - August 2021	**1.274**	**6.136**	**<0.001**
August 2020 - September 2021	0.188	1.041	0.999
September 2020 - May 2021	**-1.419**	**-5.951**	**<0.001**
September 2020 - June 2021	**-1.224**	**-4.167**	**0.003**
September 2020 - July 2021	-0.559	-2.591	0.376
September 2020 - August 2021	**0.848**	**3.912**	**0.008**
September 2020 - September 2021	-0.238	-1.248	0.996
May 2021 - June 2021	0.195	0.669	1.000
May 2021 - July 2021	**0.860**	**3.689**	**0.018**
May 2021 - August 2021	**2.267**	**9.381**	**<0.001**
May 2021 - September 2021	**1.181**	**5.435**	**<0.001**

June 2021 - July 2021	0.665	2.350	0.553
June 2021 - August 2021	**2.072**	**7.132**	**<0.001**
June 2021 - September 2021	**0.986**	**3.646**	**0.021**
July 2021 - August 2021	**1.406**	**6.473**	**<0.001**
July 2021 - September 2021	0.320	1.668	0.943
August 2021 - September 2021	**-1.086**	**-5.543**	**<0.001**

Supplementary Information Table 9: Schedule of picture trials (7 pictures per trial) and subsequent sampling of brood combs for later investigations. Picture intervals partly match with brood termination protocols of OECD guideline 75 [1]. Note that brood fixation day (BFD) is the day of egg laying according to [1].

Picture No.	Days relative to capping (± 1 day)	Expected brood stadium	OECD 75 [1]
1	-2	Old larvae	-
2	0	Sealed brood	-
3	2	Sealed brood	BFD +10
4	4	Sealed brood	-
5	6	Sealed brood	-
6	8	Sealed brood	BFD +16
7 sampling of brood combs	10	Sealed brood	-

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Chapter IV

Heritability of *Apis mellifera* recapping behavior and suppressed mite reproduction as resistance traits towards *Varroa destructor*

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Heritability of *Apis mellifera* recapping behavior and suppressed mite reproduction as resistance traits towards *Varroa destructor*

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The selection of honeybee strains resistant to the ectoparasitic mite *Varroa destructor* is generally considered as one of the most sustainable ways of coping with this major bee parasite. Thus, breeding efforts increasingly focus on resistance parameters in addition to common beekeeping traits like honey yield and gentleness. In every breeding effort, the success strongly depends on the quantifiability and heritability of the traits accounted. To find the most suitable traits among the manifold variants to assess *Varroa* resistance, it is necessary to evaluate how easily a trait can be measured (i.e., testing effort) in relation to the underlying heritability (i.e., expected transfer to the following generation). Various possible selection traits are described as beneficial for colony survival in the presence of *Varroa destructor* and therefore are measured in breeding stocks around the globe. Two of them in particular, suppressed mite reproduction (SMR, *sensu lato* any reproductive failure of mother mites) and recapping of already sealed brood cells have recently gained increasing attention among the breeders because they closely resemble resistance mechanisms of some *Varroa*-surviving honeybee populations. However, it was still unknown whether the genetic background of the trait is sufficient for targeted selection. We therefore investigated the heritabilities and genetic correlations for SMR and REC, distinguishing between recapping of infested cells (RECinf) and all cells (RECall), on an extensive dataset of Buckfast and Carniolan stock in Germany. With an accessible h^2 of 0.18 and 0.44 for SMR and an accessible h^2 of 0.44 and 0.40 for RECinf, both traits turned out to be very promising for further selection in the Buckfast and Carnica breeding population, respectively.

KEYWORDS

breeding, selection, performance testing, genetics, heredity, honeybees

Introduction

Modern breeding approaches in the honeybee realm are based on the needs of apiculture. The ectoparasitic mite *Varroa destructor* [Anderson & Trueman (Mesostigmata: Varroidae)] plays a key role for honeybee health by harming its host *Apis mellifera* [Linnaeus (Hymenoptera: Apidae)] through direct feeding and virus vectoring (1–3). Thus, the mite presents unambiguously a major task for both, apiculture (2, 3) and honeybee breeding efforts (4–6) worldwide. Currently, most colonies of *A. mellifera* managed for apiculture depend on regular miticide treatments to survive the *Varroa* infestation (2). Breeding towards resistance against this parasite seems to be the most promising and sustainable way of dealing with this problem (6, 7), although this approach does not offer an immediate solution for the global beekeeping industry (8). It rather represents a part of integrated pest management strategies to achieve a sustainable coexistence between mites and bees under beekeeping conditions (5, 9, 10).

Resistance can be scored in a broader sense in a) surviving and b) not surviving the mite infestation without treatment. Based on the fundamental idea of natural selection, this dichotomous approach has been used successfully in some untreated breeding populations (11–13). The general idea of natural selection thereby arose from the survival of mostly free-living and unmanaged colonies described in several locations around the world (12, 14–19). This selection approach proofed to be a valuable strategy, in parts acting as a role model for many breeding efforts (5, 20) and biotechnical methods of *Varroa* control (9). Under central European conditions however, it is difficult to implement this system strictly (i.e., colonies either survive or die) in larger breeding programs or management practices. Although the intensity and structure of breeding schemes differs clearly between countries, the different programs typically focus on additional desirable beekeeping traits besides *Varroa* resistance measures (5, 21–25). Thus, a more detailed resistance scoring scale is needed for comparisons among colonies already selected for other beekeeping traits. To achieve such comparability in *Varroa* resistance, selective breeding has been applied to several scorable traits which are beneficial to the health of honeybee colonies. This includes a) the proportion of mites removed by grooming, b) the share of removed injured brood cells or c) the post capping duration of broodcells (see reviews by 5, 10, 26), among others. In contrast to the mere survival of colonies, this approach aims to select the underlying mechanisms of resistance, which were found to play key roles in naturally surviving populations (14, 26, 27). Since the effects of different resistance traits are likely to sum up or act synergistically together (14), it seems reasonable to account for several resistance traits in parallel (28). Thus, various described survival mechanisms have been tested as possible selection criteria (6, 26, 28).

As for other beekeeping related traits, the selection progress is thereby highly dependent on organized breeding schemes (21), controlled mating (29) and most importantly, heritable traits (5, 8). If all of these basic requirements are met, the progress in selected traits can be substantial within a few generations (21). This applies especially when detailed knowledge on the heritability of traits is used to calculate breeding values as a guidance for selection decisions (21, 30, 31).

Mite population development (VID) and hygienic behavior are the most frequently tested traits related to *Varroa*. For both characters, significant selection effects were achieved through selective breeding in a big managed population (5, 21). Among the above-mentioned requirements, this selection progress based especially on the practicable and standardized testing protocols for both traits (21, 22). While the hygenic behavior turned out to be strongly hereditary anyway (h^2= 0.52), the comparatively low heritability of VID (h^2= 0.11) thereby seemed to be compensated by the simple testing procedure and thus extensive data base (21). On the other hand, several traits were discarded as selection criteria after a few generations, since their heritability proofed to be too low compared to the testing effort (reviewed in 5, 6, 10, 22, 26).

Three other candidate traits have frequently been associated with colony survival and are currently accounted for in breeding programs. These are a) *Varroa*-sensitive hygiene (VSH), b) suppressed mite reproduction (SMR, sensu lato) also called mite non-reproduction (MNR) according to (26), and c) the opening and recapping (REC) of already sealed brood cells (26, 27, 32, 33). As ruled out by Büchler et al. (5), any suitable selection trait needs to be both, heritable and easy to measure in practice. In case of VSH, a comparatively low heritability (h^2) of 0.18 was described (8), while tedious measurements are required for data acquisition (34, 35). Nevertheless, it seems to be an important trait for colony survival (26, 36) and was successfully selected for in some commercially breeding lines (36). Interestingly, it also contributes strongly to reproductive failure of mites (i.e., SMR) on the long-term (26, 37), although the expression of this trait is also affected by other parameters (27, 32, 38, 39) and thus shows low repeatability in individual colonies (28, 40). Likewise, the REC behavior is commonly increased in surviving colonies (27, 32, 33) and can suppress the reproductive success of mites (27, 32). Compared to the measurement of VSH, the data acquisition on SMR and REC is rather simple, since an artificial infestation is not obligatory and sampled combs can be stored in the freezer up to brood investigation (41). Hence, both traits hold great potential for effective resistance breeding if the heritability would be high enough (40). To the best of the authors' knowledge, heritability for SMR was only estimated once based on a dataset of 28 queens (42), while there is currently no estimation for the heritability of REC of *Varroa*-infested cells available. As pointed out by Eynard et al. (40), an large-scale estimation of heritability for SMR is urgently needed to use the potential of this trait more efficiently for resistance breeding. The same applies for REC of infested cells

Abbreviations: AGT, Arbeitsgemeinschaft Toleranzzucht e.V.; GdeB, Gemeinschaft der europäischen Buckfastimker e.V.; LIB, Länderinstitut für Bienenkunde e.V., Hohen Neuendorf; LLH, Landesbetrieb Landwirtschaft Hessen Bieneninstitut Kirchain; MDI, multi-drone-inseminated; MNR, mite non-reproduction; REC, recapping behavior in general; RECall, recapping behavior assessed in all cells investigated; RECinf, recapping behavior assessed in all single infested cells investigated; SDI, single-drone-inseminated; SMR, suppressed mite reproduction.

since this trait is gaining increasing attention in breeding efforts. Detailed knowledge on the heritability would also set the basis for breeding value estimation and thus enable a more targeted selection. Therefore, we estimated the heritability of SMR and REC based on an extensive dataset of Buckfast and Carniolan stock and implemented these traits in practical breeding value estimation schemes.

Materials and methods

Sources of measurement data

The majority of colonies (57% for Carnica, 100% for Buckfast) was tested between 2019 and 2021 in the framework of a nationwide selection program on suppressed mite reproduction (SMR) and recapping behavior (REC) in Germany [(43), Table 1]. In this project, several regional breeding groups and institutes jointly tested Buckfast and Carnica colonies for their expression of SMR and REC. Colonies were either full grown performance test colonies, or nuc-sized MiniPlus colonies. In the latter case, colonies were mostly headed by single-drone-inseminated queens (SDI) and partly by multi-drone-inseminated queens (MDI). Due to the limited egg laying capacity of the SDI queens, MiniPlus colonies were exclusively built up for brood investigations on REC and SMR. Full grown performance test colonies were additionally tested for common beekeeping traits (e.g., honey yield) according to the GdeB and AGT test protocols (24, 25). Within the framework of these test protocols (24, 25), selection decisions, e.g. mating choices, were made by the individual breeders.

Another data source was the predecessor project at LLH Bee Institute Kirchhain (LLH), which comprised colonies from Austria and Croatia (28). Other measurements have been deposited to BeeBreed (44) in two projects at Länderinstitut für Bienenkunde e.V. (LIB), by Dutch breeders and further breeders unrelated to the previously mentioned projects (Table 1, Figure 1). See Figure 2 for a graphical representation of the number of Buckfast colonies.

TABLE 1 Number of colonies with brood investigations from the different data sources used.

Data source	Breeding Population	
	Carnica	Buckfast
MiniPlus colonies (43)	875	1,492
Performance test colonies (43)	632	292
LLH and AGT (28)	243	–
Austria (28)	147	–
Croatia (28)	135	–
Netherlands (44)	193	–
LIB (44)	89	–
Other breeders in BeeBreed (44)	216	–
Total	**2,634**	**1,784**

Abbreviations are given in the supplements.

Brood investigations on suppressed mite reproduction (SMR) and recapping behavior (REC)

Brood combs were either investigated immediately after sampling (i.e., alive), or stored at -20°C until investigation. The occurrence of SMR was investigated in single infested cells. The expression of REC was assessed over all cells (RECall) and infested cells (RECinf) separately. Therefore, brood cells were opened separately to investigate the underside of the cell cap for signs of recapping, i.e. holes in the pupal cocoon. Afterwards, cell infestation and reproductive status of mites was examined. All investigations followed the protocol of the Research Network on Sustainable Bee Breeding (39, 41). Accordingly, reproductive failure in terms of SMR was defined as a single infested cell containing either a) no mite offspring (i.e., infertile), b) only female offspring (i.e., male missing) or c) mite offspring, which was too young in comparison to the developmental stage of the respective host bee pupae (i.e., delayed reproduction).

For the majority of full-grown colonies, up to 1000 brood cells were opened until 25 single infested cells were found (43). For MiniPlus colonies, up to 300 brood cells were opened until at least 10 single infested cells were found (43). In heavily infested colonies, more infested brood cells were analyzed. For SMR and RECinf calculations in (43), values obtained from less than 10 single infested cells were discarded. For some colonies analyzed by external contributors, other standards might have been applied.

For Carnica colonies, the clearance rate in pin tests has been obtained following the AGT protocol (25).

Pedigree information

Respective pedigree information for each queen were derived from the Beebreed-Database (44) for Carniolan stock or the Pedigree-Database (45) for Buckfast stock, respectively. For the calculation of genetic parameters, a sub-pedigree was created which contained all colonies investigated and their complete ancestor trees. Thus, for Carnica 3.250 colonies and for Buckfast 2.592 colonies were added to complete the pedigree.

Breeding model

Models of SMR, RECall and RECinf have been set up as mixed-linear models with a direct genetic effect (effect of the worker community), a maternal genetic effect (effect of the queen), a fixed effect of the investigation series and country (MiniPlus, LLH/AGT, Austria, Croatia, Netherlands, LIB, other for Carnica; MiniPlus and performance tested colony for Buckfast) and a random effect, analogous to (21). As the colonies have not been organised in comparative testing apiaries (some breeders sent in brood samples of only one colony per apiary), a fixed apiary effect was out of the question.

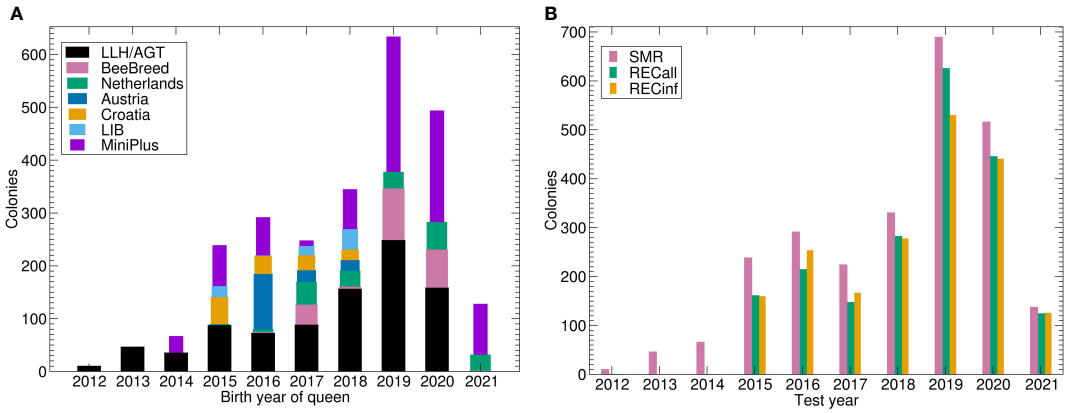

FIGURE 1
Number of colonies under brood investigation for the Carniolan breeding population. **(A)** Number of colonies tested by birth year of queen and data source. LLH/AGT comprises both performance tested colonies within (43) and previous projects of LLH and AGT. **(B)** Number of phenotypes by trait and test year. Note that the number of SMR is higher because REC was only recorded for a fraction of colonies. Abbreviations are given in the supplements.

Parameter estimation

The genetic parameters (i.e., heritabilities and genetic correlations) have been calculated with programs of the BLUPF90 series (46) in an iterative process, as follows.

First, AIREML was run. If AIREML converged and the parameters were not on the boundary (either the genetic variance vanished, or the residual variance vanished), the result was verified with a subsequent REML run. If AIREML was not successful, different start parameters were used. If AIREML was still not successful with these parameters, REML was run. The results of REML were then used in a subsequent AIREML run to confirm the result and obtain the standard errors of the parameters.

Initially, single-trait models were run to estimate the genetic variance, the genetic covariance between the direct and maternal effect and the residual variance. Then, two-trait models were composed of each combination of one-trait models with the final parameters.

From genetic and residual variances for both direct and maternal effects, the workers' effect heritability was calculated as $h^2_w = \sigma^2_{AW}/\sigma^2_{AP}$, where σ^2_{AW} is the additive variance of the workers' effect and σ^2_{AP} the phenotypical variance calculated as $\sigma^2_{AP} = a_{ss}\sigma^2_{AW} + \sigma^2_{AQ} + \sigma_{AQAW} + \sigma_E$, where a_{SS} is the additive genetic relationship between two drone producing queens reared from the same colony, σ^2_{AQ} is the additive variance of the queen effect, σ_{AQAW} is the genetic covariance of queen's and workers' effect and σ_E is the residual variance. The queen's effect heritability was calculated as $h^2_Q = \sigma^2_{AQ}/\sigma^2_{AP}$, the heritability of the selection criterion was calculated as $h^2_{SC} = (\sigma^2_{AQ} + \sigma^2_{AW} + 2\sigma_{AQAW})/\sigma^2_{AP}$. The accessible heritability was calculated as $h^2_A = a_{ss}h^2_{SC}$. See Hoppe et al., 2020 (21) for more details.

Breeding values estimation and validation

For the breeding values estimation, the pedigree was extended to contain siblings (and their siblings, iteratively) of colonies.

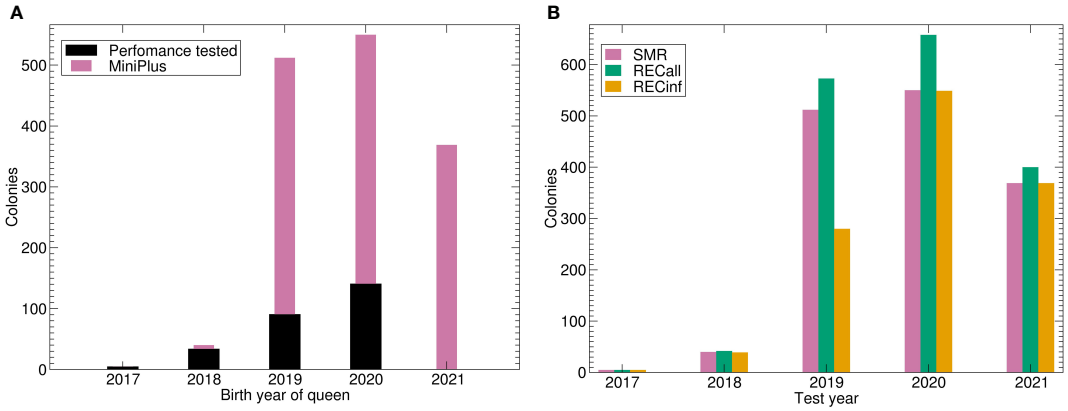

FIGURE 2
Number of colonies under brood investigation for the Buckfast breeding population. **(A)** Number of colonies tested by birth year of queen and data source. **(B)** Number of phenotypes by trait and test year. Abbreviations are given in the supplements.

Additionally, a pedigree entry for the colony was added, which represents the expectation value of an offspring queen. To calculate the breeding values, BLUPF90 was used (46).

The genetic trend shows the yearly averages of all breeding values per year obtained from colonies with measured SMR phenotypes.

To estimate the predictive power of the breeding model, the following validation procedure was used which relates the breeding values calculated ignoring the phenotypes of the test year (and all subsequent years) with the phenotypes of the test year. The breeding values were estimated with the full pedigree, while the phenotypes were discarded. As a first measure, the Pearson correlation coefficients between the breeding values and the phenotypes (adjusted for the fixed effect) were calculated. As a second measure, all tested colonies of the test year were sorted by their breeding values and split into four quartiles. Then, the average phenotype (adjusted for the fixed effect) of each quartile was calculated. This process was iterated for test years 2017 to 2022 for the Carncia population and 2019 to 2022 for the Buckfast population.

Results

Carnica

The calculation of genetic parameters was feasible for all single-trait and double-trait models. All investigated traits show comparatively high heritabilities (Table 2).

The worker heritabilities h^2_w for SMR and pintest show a peculiarity of heritabilities larger than 1. This is possible because "the worker" is not a single animal but a collection of individuals. However, this heritability is not accessible to selection because one can only use individual animals for selection and not the full community of workers. This effect is compensated for by the correction formula of the accessible selection (21).

The heritability is highest for pintest, which puts the potential for selection progress into perspective. The heritability is very similar for SMR and both recapping traits, regarding the standard error.

The genetic correlations between the queen and worker effect are strongly negative, especially for SMR (Table 3).

The genetic correlation (see Table 3) is highest between both recapping traits. SMR can be considered as not correlated to both recapping traits, the low values are overshadowed by the standard errors. Interestingly, the pintest is correlated to SMR and both recapping traits with a medium correlation coefficient, which presents a partly paradox finding.

The trend of the phenotypes (Figure 3A) shows a strong upward trend for SMR, while REC starts low, peaks in 2018 and decreases again. The genetic trend (Figure 3B) shows a similar picture. For SMR, there is a strong genetic trend upwards. Apparently, the stock was successfully selected for SMR. The genetic effect of both recapping traits starts lower than the current level, respectively. The trend is very similar for both Recapping traits, not surprisingly because of the high genetic correlation. In comparison between the two Recapping traits, the genetic trend is stronger for RECinf.

See Figure 4 for the breeding value validation charts. Comparing the correlations, i.e., predictivity of breeding values, it is best for RECinf and also very good for RECall, but comparatively poor for SMR. For all traits, the best quartile has by far the highest phenotypes, whereas the lower quartiles do not show large differences, i.e., the higher breeding values are the more predictive. The y-axis-scales reveal that the phenotypical differences between the quartiles are very high for RECinf, high for RECall and low for SMR. The difference between the highest and lowest quartile for SMR is less than 3 percentage (Figure 4A).

The high predicitvity of the recapping traits shows that it would be possible to effectively select for RECinf and RECall, but apparently, this has not been done in the investigated population. For RECinf, the highest quartile is 20 percent points higher than the lowest (Figure 4B). For RECall, the highest quartile is by 14 percent points larger than the other three (Figure 4C).

Buckfast

The calculation of genetic parameters for the Buckfast population was feasible for all traits including the two-trait models. This is a remarkable result because the number of phenotypes was considerably smaller than for the Carnica population and it does not span over generations (Table 1; Figure 2A).

The heritabilities for SMR and RECall in the Buckfast population are smaller than in the Carnica population, while for

TABLE 2 Heritabilities in Carnica colonies.

	Trait			
	SMR	RECall	RECinf	Pintest
Accessible h^2_A	0.44 (± 0.06)	0.46 (± 0.06)	0.40 (± 0.06)	0.72 (± 0.08)
Selection Criterion h^2_{SC}	0.82 (± 0.11)	0.86 (± 0.12)	0.76 (± 0.12)	1.36 (± 0.16)
Queen h^2_Q	0.57 (± 0.11)	0.48 (± 0.12)	0.30 (± 0.10)	0.47 (± 0.14)
Worker h^2_W	1.22 (± 0.10)	0.51 (± 0.09)	0.44 (± 0.09)	1.17 (± 0.16)
Queen/Worker r_{QW}	−0.90 (± 0.03)	−0.44 (± 0.13)	−0.36 (± 0.20)	−0.64 (± 0.10)

For the calculation of the different types of heritabilities and correlations see (21). Standard errors are given in brackets. Abbreviations are given in the supplements.

TABLE 3 Genetic correlations between traits in Carnica colonies.

Trait	RECall	RECinf	Pintest
SMR	*0.078* (± 0.12)	*-0.064* (± 0.12)	0.42 (± 0.11)
RECall		0.79 (± 0.06)	0.38 (± 0.10)
RECinf			0.45 (± 0.11)

Correlation coefficients are given in italics if the confidence interval (given by AIREML standard error of the correlation) contains zero, i.e., if the correlation is not significantly different from zero. Standard errors are given in brackets. Abbreviations are given in the supplements.

RECinf they are slightly higher (see Table 4). All traits are dominated by the worker effect.

The genetic correlation between queen and worker effect is negative for SMR, similar to the parameters in the Carnica population. For both recapping traits the genetic correlation between queen and worker effect is positive. However, the standard errors are so large that it is not considered significantly distinguishable from zero.

The genetic correlation between both recapping traits is even higher than in the Carnica population (Table 5). It is so close to one that it suggests it may be not possible to select for RECinf without also increasing RECall. The correlation from SMR to RECall is slightly negative regarding the standard error at the same amount. The genetic correlation of SMR to RECinf is effectively zero at this level of standard error.

Although only 5 years are represented, a positive genetic trend is visible for all traits (Figure 5). These trends are more apparent in the genetic trends than in the phenotypes where they are nearly invisible. The genetic trend is much stronger for RECinf and RECall than for SMR.

The predictivity of breeding values (correlations in Figure 6) is highest for RECall (0.235), followed by RECinf (0.160), and lowest for SMR (0.098). In comparison to the results in the Carnica population, the ranking among the traits RECall and RECinf is reversed. The predictivity of SMR and RECall is somewhat higher than in the Carnica population, whereas the predictivity for RECinf is much lower.

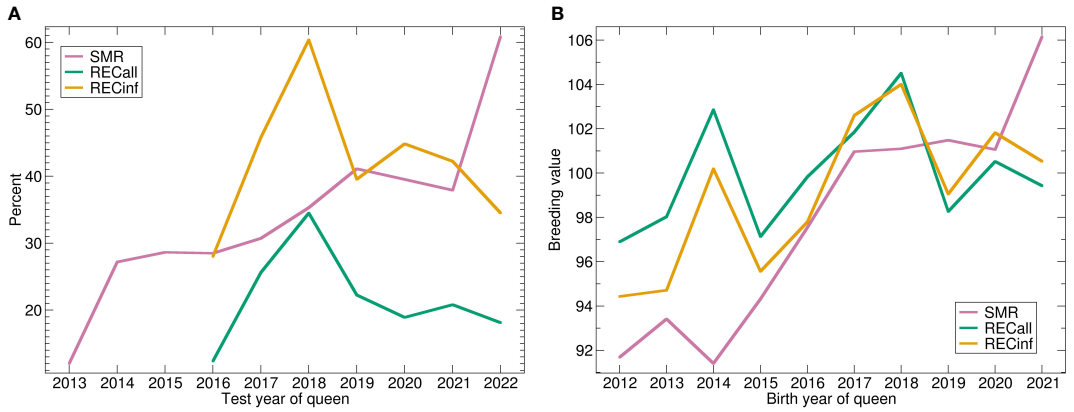

FIGURE 3
(A) Phenotypic and **(B)** genetic trends for SMR, RECall and RECinf in the Carniolan population. Abbreviations are given in the supplements.

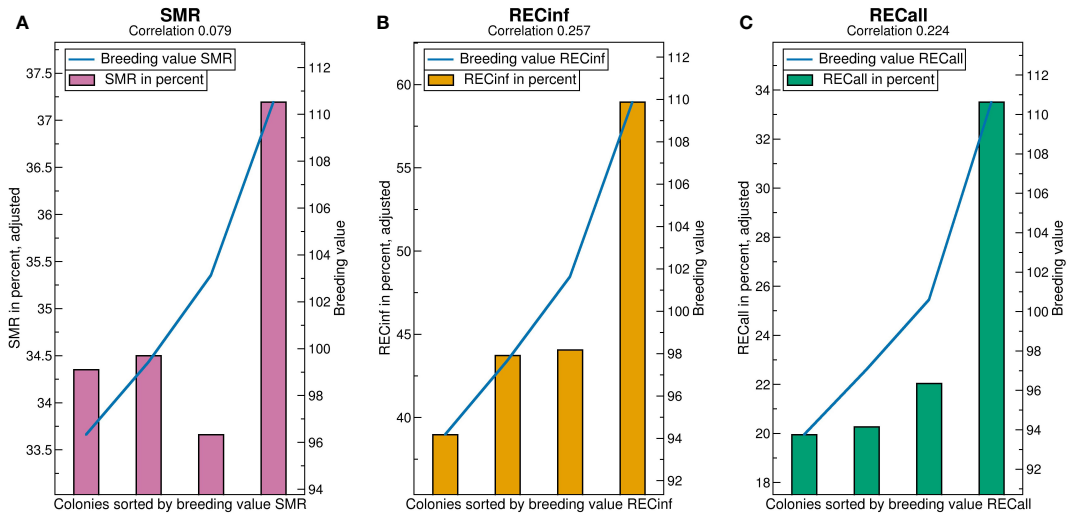

FIGURE 4
Validation charts for **(A)** SMR, **(B)** RECinf and **(C)** RECall breeding values in the Carnica population. Abbreviations are given in the supplements.

TABLE 4 Heritabilities in Buckfast colonies.

	Trait		
	SMR	RECall	RECinf
Accessible h^2_A	0.18 (± 0.07)	0.33 (± 0.07)	0.44 (± 0.09)
Selection Criterion h^2_{SC}	0.34 (± 0.14)	0.62 (± 0.13)	0.83 (± 0.18)
Queen h^2_Q	0.25 (± 0.13)	0.12 (± 0.08)	0.16 (± 0.12)
Worker h^2_w	0.32 (± 0.11)	0.22 (± 0.09)	0.32 (± 0.12)
Queen/Worker r_{QW}	–0.66 (± 0.79)	*0.21 (± 1.9)*	*0.12 (± 1.11)*

For the calculation of the different types of heritabilities and correlations (21). Standard errors are given in brackets. Abbreviations are given in the supplements.

TABLE 5 Genetic correlations between traits in Buckfast colonies.

Trait	RECall	RECinf
SMR	*-0.18* (± 0.18)	*-0.026* (± 0.27)
RECall		0.96 (± 0.02)

Correlation coefficients are given in italics if the confidence interval (given by AIREML standard error of the correlation) contains zero, i.e., if the correlation is not significantly different from zero. Standard errors are given in brackets. Abbreviations are given in the supplements.

For SMR, the low quartile separates from the rest, as opposed to the Carnica population (Figure 4A) where the highest quartile stands out. The difference between the highest and lowest quartile is about 8 percent points higher than in the Carnica population. For RECall and RECinf, the result resembles the results found in the Carnica population, where the highest quartile is considerably higher than the others. The difference between the highest and lowest quartile is about 14 percent points for both.

Discussion

We have demonstrated that it is possible to estimate genetic parameters for SMR, RECinf and RECall and that the derived breeding models are valid despite relatively few assessed generations. This sets a valuable yardstick on how many colonies are necessary to understand the genetic properties of new traits. In addition, the heritabilities of all traits are relatively large showing good selection potential in both populations. However, the predictivity of breeding values for SMR is quite low, which is in concordance with the reported low repeatability of SMR measurements (28). This results in an interesting paradox, that although measuring SMR is comparatively inaccurate, selection for this trait is still effective. Similar results have been shown in the BeeBreed Carnica population selected for low VID (21). Similar to the values presented for SMR in the present study, the breeding values for this trait hold a very low predictivity of 0.081 (21). Despite this, breeding value based selection resulted in substantial genetic progress for VID (21). Thus, regardless of their low predictivity, the selection based on breeding values rather than raw measurements of phenotypes will most probably also advance the selection on SMR.

Hence, we have shown that SMR, RECinf and RECall can be increased by targeted selection, and indeed have increased already in few breeding generations. However, it must be noted that this alone does not guarantee complete resistance to *Varroa*, and finally improved colony vitality and decreased necessity of *Varroa* management practices in the future. Resistance *per se* is a varying combination of several possible traits acting together in the respective environment (5, 47) thereby reducing the reproductive ability of the parasite (26). In contrast to easily measurable traits like honey yield,

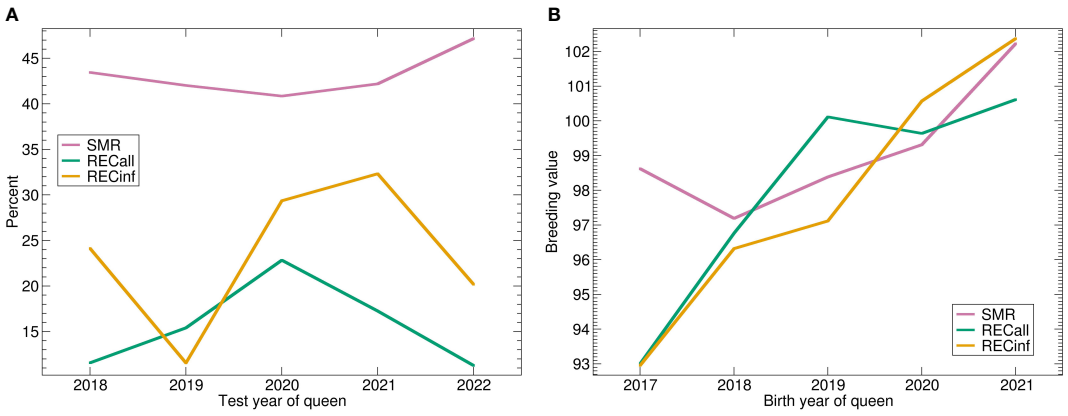

FIGURE 5
(A) Phenotypic and **(B)** genetic trends for SMR, RECall and RECinf in the Buckfast population. Abbreviations are given in the supplements.

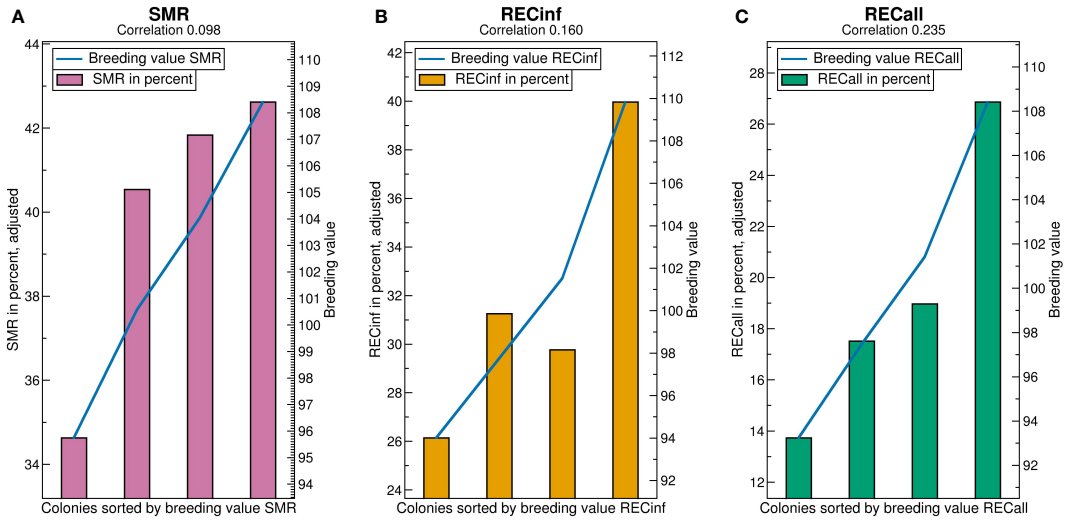

FIGURE 6
Validation charts for **(A)** SMR, **(B)** RECinf and **(C)** RECall breeding values in the Buckfast population. Abbreviations are given in the supplements.

the generic term *Varroa* resistance is therefore difficult to quantify in an accuracy required for selective breeding on a larger scale, where typically also other selection parameters are accounted for (5, 21–25, 45). By adapting measurable traits like SMR and REC from surviving populations (14, 26, 27, 32, 33) to bigger managed populations under targeted selection, we aim to include more aspects of resistance into locally adapted and selected stocks. In case of SMR and REC, average values of 45% SMR and 55% RECinf were recently reported for surviving populations (reviewed by (33)) which seems reachable for both breeding populations investigated in the present study. However, it is very important to avoid a situation in which breeds show high values of SMR and REC but are less adapted to other factors or are unmanageable for beekeeping. The estimation of genetic correlations between SMR, RECinf and RECall on the one hand and the traditional breeding traits on the other hand serves to foresee problems of this kind. Therefore, a continuation of brood investigation as part of the performance test is highly recommended, since such information cannot be obtained from MiniPlus colonies.

In addition, it seems to be important to account for multiple resistance traits in the future. Besides their possibly varying importance discussed above, several of these traits appear to be linked and thus selected in parallel. Despite the fact that only SMR was measured in the beginning, the genetic trend for both REC traits also started lower than the current level. This indicates that the genetic progress obtained for SMR also unintentionally led to an increase in REC as well, before REC parameters were even accounted. Likewise, the small range of SMR breeding values in the Carnica population indicates that the strong genetic trend for this trait is partly dependent on the selection of other causally linked parameters, e.g., low *Varroa* infestation development (VID).

Also, the traditional breeding traits do not fully represent what is needed to assess vitality in the context of *Varroa* burden. It is therefore recommended to transform vitality scores already quantified in scientific studies (e.g (48).,) into regular breeding traits. As a very accurate approach in this direction, the AGT recommends the so-called "vitality test", a protocol to postpone *Varroa* treatment until a critical infestation is reached, which implies constant monitoring of the infestation level (25). However, up to now this serves mainly as an additional information for the breeders. What is lacking is an outcome variable of this "vitality test" both readily applicable to the regular breeder and expressive for the mathematical model. Here, more research is necessary.

As the Carnica population considered in this study overlaps with the BeeBreed Carnica population (21) and most performance test colonies and even some MiniPlus colonies underwent pintest, a comparison with the genetic parameters of the pintest can be made. The heritability obtained in the present study (h^2= 0.72) is much higher than in the BeeBreed Carnica population (h^2= 0.21) (21). To explain this difference, it has to be noted that there is a fundamental difference in the genetic model applied for the pintest in these calculations. In the Carnica breeding system, a fixed effect of testing apiary and year is applied. Thus, environmental effects of the test season are removed from the breeding values. This results in a high predictivity of the respective breeding values. Here, such a fixed effect could not be applied, because the brood samples were often derived from only one colony or very few colonies per testing apiary. It is known that a different definition of a fixed effect leads to very different heritability estimations. As the breeding values estimation with apiary-year fixed effects is appropriate, indicated by steep selection progress and high predictivity of breeding values (21), we conclude that the heritability for pintest estimated in the present study is artificially bloated. Thus, we may also assume that the heritabilities of SMR and recapping traits are bloated to the same extend. Consequently, it can be hypothesized that if SMR and REC were tested in an apiary-year context, its heritability would be

likewise lower and thus approximately at the level of the honey yield heritability in the Carnica population (h^2= 0.14) (21). However, this would absolutely suffice as the selection for honey yield, especially based on breeding values, has been proven to be very effective in practice (21). In fact, the negative genetic correlations between queen and worker effects for REC and SMR in the Carnica population investigated in the present study already indicate previous selection on these parameters. This is especially apparent in the strong genetic progress of SMR, while the quartile distribution for RECinf suggests that the potential for a bigger selection effect rather could be used in the future.

Despite this promising genetic background, SMR and REC measurements can be affected by various external factors (27, 32, 38, 39, 49). It thus seems to be rational to include apiary-year information in the raw data acquisition and breeding value estimation for following breeding efforts in order to increase the breeding value predictivity. In addition to apiary effects, it should be likewise accounted for variation through differences in data acquisition. Since the brood investigation methods require training and experience, the practical knowledge of investigators is likely to contribute to the variation of phenotype values. This might particularly apply for the investigation of MiniPlus colonies with SDI or MDI queens. These colonies are mostly investigated in smaller batches by private breeder groups, while samples from performance tested colonies are mostly processed by research institutions and professional investigators. Since the work of private breeders is essential for a broad genetic basis, while the additional work load of brood investigations in the season is immense, possible simplifications of the testing protocols need to be investigated. For instance, it is much easier to just score RECall (50, 51) and the question remains if, for a fraction of a population, this would be worthwhile. Again, this is linked to the estimation of genetic correlations between different traits. Another option would be brood investigation services offered by companies which evaluate SMR and REC in brood combs sent in by breeders. Similar services from professional investigators are common for morphometric analysis in the Carnica Population. Such a central evaluation of brood combs by trained investigators would not only ease the testing efforts for breeders, but also increase the accuracy of measurements. However, the question how the costs are shared among private breeders, breeding associations or the whole beekeeping community must be taken into account. Besides the promising genetic parameters of SMR and REC shown in the present study, such implementations in practicable performance testing procedures are urgently needed for successful selection. Without easy-to-apply test protocols for the practice, even heritable traits are unlikely to gain substantial genetic progress on the long-run. For example, breeding efforts for increased grooming behavior of the workers have largely stalled in Europe. Although some selection progress could be achieved in focussed breeding programs (20, 52), the heritability (h^2) of 0.16 (53) or even lower (54) seemed to be insufficient for an ongoing large scale selection with laborious testing requirements (5, 52, 55). In case of SMR and REC, both investigated populations show promising heritabilities and genetic trends for these traits, but are likewise dependent on a large-scale performance testing of colonies.

To our surprise, the calculation of genetic parameters for the Buckfast population proved less problematic than expected from the fact that only three seasons of data recording could be used and values mainly derived from MiniPlus colonies. In addition, the concept of Buckfast breeding distinctively differs in some points from the methods widely used in Carnica breeding which may lead to differences in population structure. For example, selection is solely based on performance of colonies without any form of morphometric analysis of workers and drones. This also includes the performance testing of new and mainly unselected strains derived from different subspecies of *Apis mellifera*, beside the regular testing and selection of established Buckfast lines. This might partly explain differences in the genetic correlations between queen and worker effects for REC parameters, which were negative for Carnica colonies but positive for Buckfast colonies. Normally, positive correlations indicate a situation where the trait has not been selected previously but might be accessible for targeted selection. However, given the large standard errors, this is not guaranteed in this dataset. According to the quartile distribution for SMR in both populations, the selection for high SMR values can be predicted to be more effective in the Buckfast population (lowest quartile stands out) when compared to the Carnica population (highest quartile stands out). For RECinf, this trend seems to be inverse with less effective selection for high RECinf values in the Buckfast population.

In addition, a positive genetic trend was also visible for all traits in the Buckfast population. Interestingly, these trends were not apparent in the phenotypes, which shows that selection can occur without immediately being visible in the raw phenotype data.

To our knowledge, this is the first application of breeding value estimation in Buckfast stock. In the tradition of Brother Adam, Buckfast breeding relies on the validity of the direct (phenotypic) evaluation of colonies (56) and for over a century of successful breeding has not perceived the need for any form of breeding values. However, our study showed that the basic requirements for breeding value estimation, such as a meticulous recording of ancestry (45), are more than fulfilled for the Buckfast stock investigated in the present study.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

The investigation was designed by AH, MG, and RB. Data acquisition related to (43) was performed by JO and MG, other data sources listed in Table 1 were handled by AH. Statistical analysis and calculation of genetic parameters was performed by AH. AH and MG prepared the first draft of the manuscript. In the following, RS, RB, JO, AH, and MG commented on earlier versions of the manuscript and contributed in the writing process. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Chapter V

Discussion

Ever since its host shift to *Apis mellifera* more than 100 years ago (Le Conte et al. 2020; Wilfert et al. 2016), *Varroa destructor* has been the number one scourge of global honeybee populations and apiculture. Living up to its name, the mite rapidly destroys infested colonies if they are not treated properly by beekeepers (Traynor et al. 2020; Rosenkranz et al. 2010). Nevertheless, some honeybee populations have evolved resistance traits against *Varroa destructor* by means of natural selection, enabling the colonies to cope with this parasite on their own (Luis et al. 2022; Grindrod & Martin 2021). Starting in the 1980s, the field of *Varroa*-resistance has quickly come into the focus of apicultural research and bee breeding (Le Conte et al. 2020). Since then, this glimmer of hope for a sustainable solution of the "*Varroa* crisis" has been intensively studied, yet the problem has still not been completely solved (Mondet et al. 2020a; Büchler et al. 2010; Rinderer et al. 2010).

The present work adds to a deeper understanding of *Varroa*-resistance mechanisms and provides important findings, which will be needed to streamline breeding efforts in the future. In this discussion, the most relevant results presented in Chapters 2, 3 and 4 are therefore summarised and discussed in synopsis, leading to a concluding discussion of factors altering the expression of MNR and REC as well as shaping the interaction of both traits.

As laid out by Eynard et al. (2020), neither the complexity of MNR and its underlying mechanisms nor the potential for selection of this trait are completely understood at present. This gap of knowledge stands in sharp contrast to the huge efforts already undertaken to select and breed bee strains with increased MNR expression (Von Virag et al. 2022; Eynard et al. 2020; Mondet et al. 2020a). The same applies to REC, although the role of this behaviour as a stand-alone resistance trait, as well as its suitability for targeted selection is still controversially discussed (Guichard et al. 2022; Hawkins & Martin 2021; Martin et al. 2020; Oddie et al. 2018).

The main objective of my doctoral studies was to fill these knowledge gaps by 1) identifying factors which alter the expression of MNR and REC, 2) investigating possible links between REC and mite reproduction and, based on this, 3) evaluating the benefits and constraints of targeted selection of both traits.

These extensive investigations have yielded promising results for future breeding efforts, while also highlighting crucial methodological limitations and needs for further research and improvement.

Beginning with the brood rearing of host colonies as the basic prerequisite for *Varroa*-reproduction, it was shown that brood interruptions alter the expression of MNR and REC both directly and in the long term (Chapter 2, Fig. 2 & 4). Based on this, I consecutively followed the expression of both traits, as well as the infestation levels of colonies in long-lasting field trials to investigate their variation in the light of seasonal dynamics (Chapter 3). This proved that both MNR and REC are strongly affected by seasonal factors, while REC nevertheless steadily favoured the occurrence of MNR on the cell level (Chapter 3, Fig. 1a). In addition, MNR values correlated negatively with colony infestation, underlining the positive effects of both traits for honeybee health.

Given the outer effects altering MNR and REC uncovered in Chapters 2 & 3, the heritability of both traits appear to be questionable. As described in Chapter 4, both traits nevertheless proved to be heritable to considerable degrees. Hence, MNR and REC are likely selectable in

practice, if the implications drawn in Chapters 2, 3 & 4 are considered. This milestone for applied resistance breeding laid the foundation for incorporating MNR and REC, for the first time, into large-scale breeding value estimations in Buckfast and Carnica stocks (Chapter 4, Fig. 3 & 5) which could greatly foster precise selection decisions in the future.

Thus, my studies shed light on basic mechanisms of *Varroa*-resistance and highlight their implications for practical bee breeding. The respective points are discussed in more detail in the paragraphs below. Therefore, I first discuss the state of knowledge deriving from studies on resistant populations. In the following, the factors altering the expression of different forms of MNR are discussed and the role of REC is emphasised. Finally, the relevance of the points mentioned above is discussed in the light of applied bee breeding and sustainable hive management. The major findings and implications of the present work are condensed in Figure 5.

5.1 Natural selection – A blueprint for resistance breeding

Naturally selected honeybee populations surviving their *Varroa*-infestation constitute ideal models for different forms of resistance breeding. The mere survival of colonies despite the presence of mites proves the biological relevance of their resistance traits (Büchler et al. 2010), which, whether identified or not, might thus be desirable for resistance breeding efforts.

However, the transfer of natural selection concepts into breeding schemes greatly varies, from fundamental mass selection approaches to a targeted selection of specific traits (Mondet et al. 2020a; Büchler et al. 2010; Rinderer et al. 2010).

Some basic breeding schemes have successfully adapted the concept of natural selection known from resistant populations, even without identifying the underlying traits of *Varroa*-resistance ("black box selection" [Blacquière et al. 2019; Kefuss et al. 2015; Fries et al. 2006]). In these selection approaches, colonies remain untreated to propagate surviving colonies, while heavily infested colonies mostly die and thus do not contribute to the gene pool of the breeding population (Blacquière et al. 2019; Kefuss et al. 2015; Fries et al. 2006). Hence, it remains mostly unclear which traits enable the survival of colonies, because only the outcome (i.e., healthy and alive colony or dead colony) is accounted for. Since the basis of this approach essentially reflects the famous term "survival of the fittest", most prominently used by Charles Darwin (1869) in his works on natural selection, the method was named "Darwinian black box selection" (Blacquière et al. 2019; Neumann & Blacquière 2017). Similar breeding concepts are sometimes also casually called "Bond tests" (Kefuss et al. 2004), a term referring, somewhat morbidly, to the title of the novel "James Bond: Live and Let Die" (Fleming 1954). This title can be likewise interpreted with respect to the general concept of natural selection, which is applied in completely treatment-free approaches, and eliminates poorly adapted colonies by letting them die.

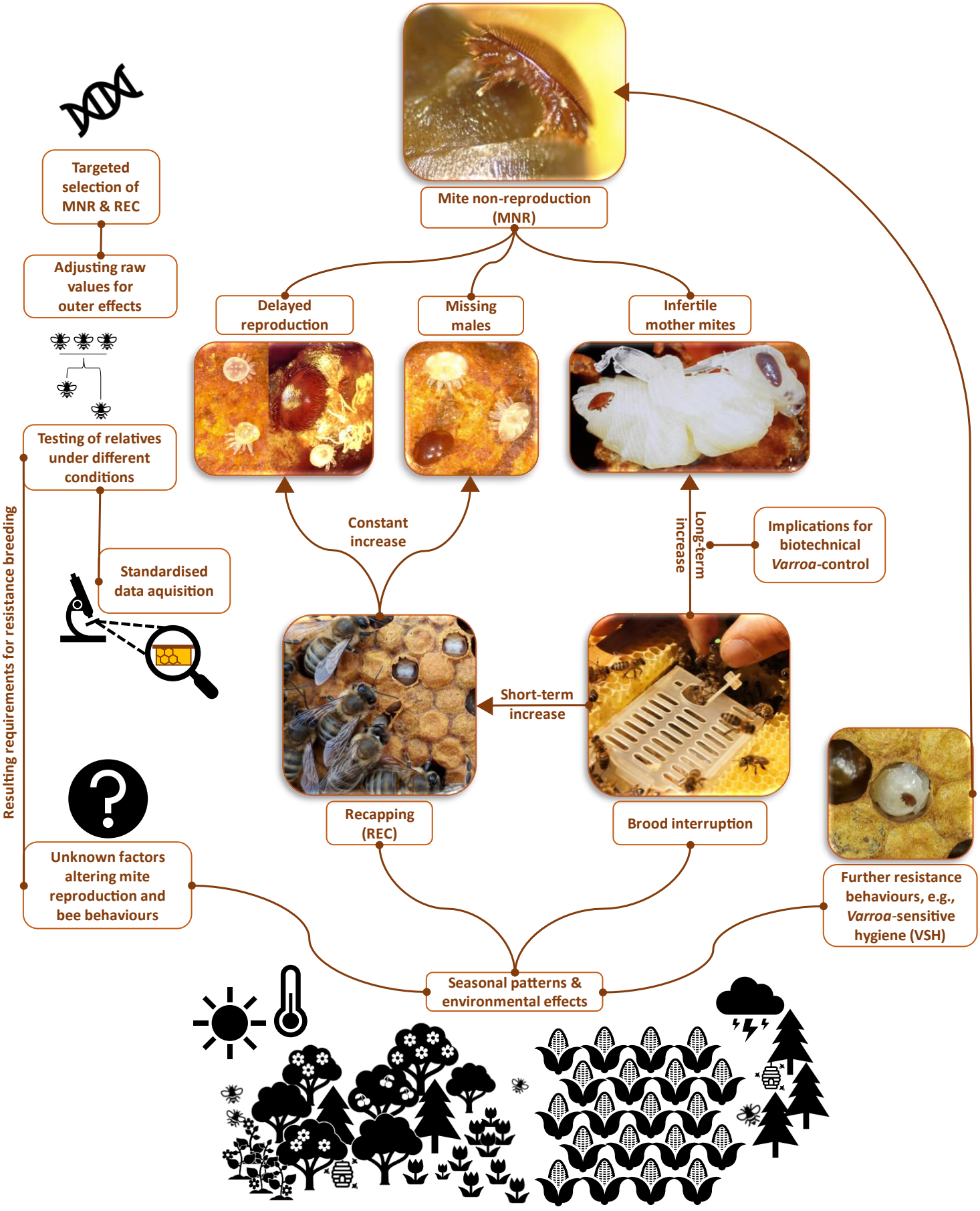

Figure 5: Schematic illustration of the major findings presented in Chapters 2, 3 & 4. Seasonal patterns and different environmental factors alter the expression of MNR and underlying bee behaviours. In this, the causes of MNR are affected differently. The share of infertile mites is increased by interruptions in brood rearing but not REC, which opens up new avenues for biotechnical *Varroa*-control. Brood interruptions also immediately increase REC but have no long-lasting effect on cells with delayed reproduction or missing males. In turn, REC constantly fosters the occurrence of delayed reproduction and missing males in *Varroa*-infested cells, yet shows no effect on infertile mites. The complex and variable origin of measured MNR and REC raw values hampers targeted selection. Resistance breeding therefore relies on standardised protocols which account for outer effects, e.g., by combining phenotypic test data from relatives and studbook information to enable a breeding-value-based selection.

Bridging natural selection and applied breeding

Although these straightforward approaches proved to be functional in the cases outlined above, they appear to be unsuitable for larger breeding populations, since vast colony losses are to be expected, and traits other than those fostering *Varroa*-resistance are lost sight of. In addition to *Varroa*-resistance, however, traits like a high honey yield or an increased gentleness of bees have a high priority in most breeding programs which follow the needs of modern apiculture (Hoppe et al. 2020; Lodesani & Costa 2003). Thus, more complex and targeted selection approaches arose, aiming for an increase in specific resistance traits in congruence with an overall progress of other beekeeping traits (Le Conte et al. 2020; Büchler et al. 2010). Traits which proved to be beneficial for colony health in naturally selected populations (see Subchapter 1.2) are thus adopted to colonies already adapted to local requirements, and are selected alongside other desirable traits. Thereby, the common acceptance of the breeding stock by beekeepers is increased.

Targeted breeding approaches nevertheless have to follow some general principles when selecting traits for an increased *Varroa*-resistance. As always in bee breeding, natural variation as well as the respective benefit of certain degrees of expression (i.e., the biological relevance of the trait [Büchler et al. 2010]) should be taken into account when evaluating traits for targeted selection (Rinderer 1986). Therefore, *Varroa*-surviving honeybee populations have been investigated extensively in order to identify such traits, since they obviously hold properties which enable them to cope with the parasite (Grindrod & Martin 2021; Mondet et al. 2020a). As discussed above, the first prerequisite for a promising targeted selection, i.e., the benefit for the colony, is thus already ensured by natural selection. In addition, any traits to be selected for need to be scorable and expressed to varying degrees in the breeding population, in order to identify queens suitable for propagation (Büchler et al. 2010). In contrast to the dichotomous model of surviving or dying colonies, this approach makes possible a finer grading of the expression of traits and thus also reveals differences within surviving populations (e.g., the percentage of recapped cells [Luis et al. 2022; Grindrod & Martin 2021]).

Ultimately, targeted selection approaches can learn from the naturally selected *Varroa*-resistant populations by identifying certain traits enabling ultimate colony survival and adapting them to the needs of apicultural and bee breeding practice.
Several such candidate traits have been described to be more pronounced in surviving populations compared to mite-susceptible ones (Luis et al. 2022; Mondet et al. 2020a; Traynor et al. 2020; Locke 2016) and were thus implemented in targeted breeding schemes with varying success (Mondet et al. 2020a; Büchler et al. 2010; Rinderer et al. 2010; see also Subchapter 1.2).

Notably, however, the survival of resistant colonies is rather ensured by a specific set of traits tailored to the respective habitat than by a single prominent trait alone (Mondet et al. 2020a; Locke 2016). Thus, resistance traits naturally selected under certain habitat conditions (i.e., locally adapted traits), can prove useless or even detrimental for colony survival under environmental conditions other than the ones in which they first evolved (Büchler et al. 2015; Meixner et al. 2015; Corrêa-Marques et al. 2002).
MNR and REC proved nevertheless to be efficient in several honeybee populations, covering different habitat types, which is why they are currently the most promising candidates for resistance breeding in mite-susceptible populations (Grindrod & Martin 2021; Mondet et al. 2020a).

In addition to such spatial differences in the importance of resistance traits, the results on the seasonal dynamics of MNR and REC presented in Chapter 3 strongly suggests temporal differences to be important as well. Thus, a diverse repertoire of resistance traits appears to be advantageous for resilient colonies in order to keep them prepared for changing conditions over time. In this context, colonies surviving their *Varroa*-infestation untreated again display valuable orientation points, since they already have mastered changing conditions with their naturally selected set of resistance traits. Thus, the frequent occurrence of MNR and REC in these populations again highlights the importance of these traits.

Besides their prominent role in naturally surviving populations, the occurrence of REC and MNR is also easy to score, e.g., as a percentage of non-reproductive mites or recapped cells (Büchler et al. 2017). Thus, assuming a sufficient heritability discussed in Chapter 4, they appear to be ideal candidate traits for applied targeted selection (Eynard et al. 2020).

Naturally *Varroa*-surviving colonies thus hold major relevance as a blueprint for targeted selection efforts; yet they also constitute a fascinating field of research in host-parasite interactions, as well as in mite and honeybee biology. Studying the coexistence of bees and mites may thus lead to a deeper understanding of natural selection mechanisms, the reproduction strategies of parasites and behavioural patterns of resistant hosts, which in turn are often crucial for successful breeding efforts in practice.
The occurrence and relevance of *Varroa*-resistance mechanisms were thus intensively studied from different points of view, gaining insights in factors altering the expression and effects of MNR and REC as described in the following paragraphs.

5.2 Mite non-reproduction (MNR) – Occurrence and origin of a major resistance trait

High levels of mite non-reproduction (MNR) as an inverse measure of a low reproductive success of *Varroa* were identified as a key driver of reduced infestation growth and colony survival in several resistant honeybee populations (Martin et al. 2020; Mondet et al. 2020a; Harbo & Hoopingarner 1997). Although having been accounted for decades (reviewed in [Mondet et al. 2020a]), this connection has still remained the focus of research efforts to this day. This long-lasting research history is rooted in the complex and diverse mechanisms behind MNR, making raw values hard to measure and interpret (see Subchapter 5.4 & Chapter 4, Fig. 3 & 5). Overall, MNR constitutes rather the outcome of several combining factors than one single trait (Grindrod & Martin 2021; Mondet et al. 2020a; see also Tab. 1 & Fig. 5). Thus, MNR in one population might be caused by one set of factors, and by different factors entirely in another population. For example, MNR could be induced by brood cues in one case (Scaramella et al. 2023), while increased VSH behaviour might be the underlying cause in another (Harbo & Harris 2005). Likewise, the mechanisms underlying the failure of mother mites might also differ on the colony level – and even on the cell level within one and the same colony. Thus, whenever accounting for MNR, it is important to keep in mind that several factors can add up to the reproductive failure measured, which, correspondingly, might be altered in a variety of ways. This matches reports of a low repeatability of MNR values (Büchler et al. 2020a), suggesting that external factors had a stronger effect on phenotypic MNR values than genetic predispositions (summarised in Subchapter 1.2.1). Eynard et al. (2020) likewise described a low repeatability when measuring MNR values at long intervals (i.e., on a monthly basis). However, the repeatability was at least modest when measuring in shorter succession (i.e., at 10 day intervals). These manifold factors possibly altering MNR therefore

appear to have upsides and downsides for applied research: many pathways might lead to the desired reduction of mite reproduction, yet the underlying causes often remain a "black box" when measuring this trait. Hence, a more detailed knowledge of factors leading to MNR would greatly foster the understanding and usage of values thus measured.

Factors leading to mite non-reproduction

In this context, effects of adult bee behaviours (Oddie et al. 2018; Harbo & Harris 2005), as well as host brood traits (Scaramella et al. 2023) appear to be most effective in reducing the reproductive success of mites. Both cases might also add up synergistically when bee behaviours lead to mismatches in the semochemical orientation of the parasite (Oddie et al. 2018; Frey et al. 2013), thereby perturbing its delicate reproductive cycle (summarised in Chapter 1). Beside this, the role of outer effects such as colony condition or seasonality in the occurrence of MNR remains mostly the subject of speculation. However, recent findings of Tison et al. (2022) have revealed that the expression of VSH depends on seasonal changes of work force capacities within the colony (summarised in Subchapter 1.2.2). Thus, it is reasonable to assume that MNR expressions would likewise depend on such changes, since VSH was reported to contribute considerably to MNR (Harbo & Harris 2005). In addition, other MNR-inducing behaviours, even though yet unknown, likely are subject to the same principle of work force allocation. As described in Chapter 2, brood interruptions, also, fostered the occurrence of MNR by decreasing the reproductive success of mites (Chapter 2, Fig. 2). Beside the foraging behaviour studied by Tison et al. (2022), the brood care performed by nurse bees reflects another huge field of worker bee duties (Winston 1987) and thus is likely also relevant for the allocation of work force capacities towards resistance behaviours. In addition, given the strong dependence of *Varroa* on the brood of its host, the absence or shortage of honeybee brood is an obstacle for *Varroa*-reproduction *per se* (summarised in Subchapter 1.1). Even in the case of reduced (i.e., not completely stopped) brood activity, mites would find fewer cells suitable for invasion. This would lead to a higher proportion of multi-infested cells with an overall reduced reproductive success of mother mites and lower mite population growth (Donzé et al. 1996; Nazzi & Milani 1996; Donzé & Guerin 1994). The results on brood infestation after interruptions of brood rearing presented in Chapter 2 (Fig. 5) clearly show such a mitigating effect on the development of *Varroa*-infestation levels and emphasise the correspondingly better health status of the respective host colonies. Similar durations of host brood interruption, with a corresponding prevention of mite reproduction, are frequently found after natural swarming (Seeley & Smith 2015; Winston 1987), which, by itself, has often been discussed as a resistance trait of untreated colonies (Loftus et al. 2016; Seeley & Smith 2015).

Furthermore, previous studies found a decreased reproductive success after artificially prolonged dispersal phases of up to 12 weeks (Stürmer & Rosenkranz 1994). The more field-realistic durations of brood absence (i.e., up to 30 days) presented in Chapter 2 proved however sufficient for decreasing the reproductive success of mites in following brood cycles (Chapter 2, Fig. 2). Thus, brood breaks, either induced by beekeepers or caused by natural swarming events, increase the occurrence of MNR beyond the mere duration of brood absence and therefore bring long-lasting beneficial effects for infested colonies. However, this suppressing effect on mite reproduction depends on the duration of previous brood interruptions, and decreases over time. This recovery of mite reproduction likely reflects the replacement of old mites, which experienced the brood interruption, by young mites hatching afterwards on a population scale. Fitting well to the commonly assumed life expectancy of *Varroa* (Martin & Kemp 1997; Fries & Rosenkranz 1996), the MNR values of colonies which

underwent a brood interruption and control colonies with constant brood rearing did not differ from each other after three consecutive cycles of unrestricted brood rearing (Chapter 2, Fig. 2).
Notably, MNR values were significantly increased as early as 10 days after caging of queens for brood interruption (Chapter 2, Fig. 2). Hence, the dispersal phase of these mites was unaffected by the brood interruption, as they had already invaded the brood cells in advance. The sharp immediate increase in MNR thus must have been induced during the reproductive phase of the respective mother mites. Since at this time (10 days after caging), all brood cells had reached the capped stadium, the need for nursing activity sharply dropped. Hence, a work force allocation towards resistance behaviours, as discussed above, appears to be the most likely cause for the spontaneous increase in MNR expression. In line with this, the recapping activity indeed sharply increased during the brood interruption (Chapter 2, Fig. V) and declined again to normal levels after new larvae were present in the brood nest (Chapter 2, Fig. 4). Although no direct effect of REC was evident, MNR could likewise be caused by an correspondingly increased removal of infested brood cells (VSH, summarised in Subchapter 1.2.2), since the behavioural patterns of REC partly overlap with VSH and are thus often seen as a proxy for this trait (Martin et al. 2020; Mondet et al. 2020a). In fact, a sharp increase in VSH might have completely overshadowed possible effects of REC on MNR, since VSH was frequently suspected to target cells with successfully reproducing mothers (Mondet et al. 2020a; Oddie et al. 2018). After their removal, these cells thus would not have been found in the subsequent brood investigations for MNR and REC. Even though some studies reported no such targeted removal of fertile cells (Sprau et al. 2021; Harris et al. 2010), it is likely that the removal of infested brood cells is expressed with some variability and follows a gradient of several triggers (Martin et al. 2020). Recent findings of Sprau et al. (2023) support this hypothesis, since they found live mites to be removed more frequently from brood cells than dead (i.e., infertile) mites which were still removed more often than inorganic objects and control cells. In line with this, brood interruptions increased MNR values only by means of higher shares of infertile mothers in the brood (Chapter 2, Fig. 3a), while the occurrence of delayed reproduction and missing males did not differ from the control group (Chapter 2, Fig. 3b, c). Thus, an increased removal of successfully reproducing cells (i.e., targeted VSH) might have led to the increased levels of remaining infertile mites. However, a simple dichotomous behaviour (i.e., removing successfully reproducing mites while leaving unsuccessfully reproducing mites untouched) would have likewise increased the shares of cells with delayed reproducing daughters and missing males. Since only the share of infertile mites was increased during brood interruptions (Chapter 2, Fig. II), VSH appears rather to be elicited by graded triggers, as supported by the findings of Sprau et al. (2023) and well known for other threshold dependent worker behaviours (Pankiw & Page 2000). Thus, cells with successfully reproducing mites indeed appear to elicit VSH most frequently, while mites with otherwise failed reproduction might be removed to a lesser extent (e.g., by few worker bees with lower thresholds for the yet unknown triggers) and thus do not differ from infertile (including dead) mites, which appear to provide the least reason for removal.

While the exact pathway leading to increased shares of infertile mites remains subject to speculation, the results presented in Chapter 2 clearly prove that brood interruptions decrease the reproductive success of mites by increasing the occurrence of infertile mothers rather than fostering the other causes for MNR.

Seasonal variation in mite non-reproduction

In addition to the apparent effects of brood interruption, even the control group showed some temporal variation in the occurrence of MNR over the study period of two months (Chapter 2, Fig. 2). This points to an additional seasonal variation of MNR, fitting earlier reports of (Otten 1991). By the subsequent study presented in Chapter 3, this seasonal variation was confirmed based on long-term measurements of MNR and its underlying causes (Chapter 3, Fig. 1). In this, the effects of seasonal brood interruptions (e.g., in winter) and corresponding work force allocation described above appear to add to a set of factors like changing nectar flows (Tison et al. 2022) and differences between summer and winter bees (Otten 1991) which jointly modulate the reproductive success of mites over the course of the active bee-season. Notably, the pattern of MNR expression over the active bee-seasons of 2020 and 2021 (Chapter 3, Fig. 1) resembles earlier findings of Otten (1991) from the years 1988 and 1989, while the reproductive success of mites differed considerably in 2019. This likely was related to differences in nectar flows and demographic changes of worker bees as discussed above, yet cannot be traced back to the respective causes. Although the exact causes for this differing pattern remain unclear, the differences between years as well as months strongly suggest that both temporal and spatial factors need to be accounted for when comparing MNR values of different colonies. Besides scientific investigations in different populations, this especially holds importance when comparing MNR values in the realm of bee breeding described in the following Subchapter 5.4.

Mite non-reproduction overall results from different factors

While the occurrence of MNR overall was strongly variable on a seasonal scale, it nevertheless proved to be constantly fostered by REC. As opposed to the effects of brood interruptions, REC however only increased the occurrence of delayed reproduction and missing males on cell level, while the occurrence of infertile cells remained unaffected (Chapter 3, Fig. 1).
Therefore, the different causes of MNR (i.e., infertility, delayed reproduction and missing males) are altered by different factors (Fig. 5) as shown for brood interruptions in Chapter 2 and REC in Chapter 3, which once again underlines the complexity of mechanisms behind this major resistance trait. This also explains the varying shares of infertile mothers, delayed reproduction and missing males reported in different resistant populations (Scaramella et al. 2023; Mondet et al. 2020b), since host colonies and mites overall face differing factors such as habitat conditions and resistance behaviours.

Thus, the occurrence of infertile mothers, delayed reproduction and missing males should be considered separately when investigating *Varroa*-resistance in different populations, since they are apparently altered by different factors which nevertheless eventually lead to the same outcome: MNR.

5.3 Recapping of brood cells (REC):

Key mechanism or failed resistance behaviour?

The uncapping and recapping of sealed brood cells (REC) is a long-known behaviour of honeybees which has gained increasing attention in recent years (Mondet et al. 2020a). In this, it is either seen as a separate resistance trait, leading to a reduced reproductive success of mites (MNR) on its own (Oddie et al. 2018), or is rather interpreted as a graded reaction to infested cells which was not sufficiently strong to end up in VSH (Martin et al. 2020; Aumeier et al. 2000). This disagreement on the role of REC derives from contradictory findings on the

relevance of REC on the colony level, either showing an suppressing effect on *Varroa*-reproduction (Oddie et al. 2021; Buchegger et al. 2018) or not (Büchler et al. 2020a; Martin et al. 2020). The same applies for more detailed investigations on the level of individual cells, which aimed to gain a deeper insight into the direct effects of REC by excluding possible interference factors present on the colony level (Guichard et al. 2022; Hawkins & Martin 2021; Oddie et al. 2018). Thus, the relevance of REC for naturally selected populations and targeted breeding efforts is still under debate, keeping it in the focus of ongoing investigations (Dall'Olio et al. 2022; Mondet et al. 2020a).
Indeed, the results gained in the present work (Chapter 2 & Chapter 3) likewise contradict each other with respect to the relevance of recapping at first glance, again underlining the complexity of resistance traits.

The importance of recapping over time
While no direct effect of REC on MNR was found in the study described in Chapter 2, the results presented in Chapter 3 prove that REC can decrease the reproductive success of *Varroa* significantly and continuously (Chapter 3, Fig. 1). As suspected before (Hawkins & Martin 2021), the importance of single behaviours for *Varroa*-reproduction however might be easily overshadowed by comparably stronger effects, e.g., the consequences of brood interruption discussed in detail above (Subchapter 5.2). Thus, the long-term investigations of MNR and REC presented in Chapter 2 are likely to paint a clearer picture of the overall potential of REC, since the data covers manifold real-life conditions over time (e.g., changes in brood amount, temperature or honey flow due to seasonal variations).
In contrast, most of the earlier studies investigated the possible effect of REC on MNR based on single samples, displaying only temporal snap shots of the apparently complex interrelationships of *Varroa*-resistance traits (Hawkins & Martin 2021; Büchler et al. 2020a; Buchegger et al. 2018; Oddie et al. 2018). In line with this, Guichard et al. (2022) described contradictory results in subsequent samples gained from the same colonies, since REC increased the occurrence of MNR in one sample set, while this was not the case in the other. REC, therefore, appears indeed to be a stand-alone resistance trait, although the importance of this trait in the reduction of reproductive success of mites may vary over time. Overall, this fits the observations of resistant populations displaying various traits at once as discussed in detail in Subchapter 0. The frequent occurrence of increased REC in such populations (Luis et al. 2022; Grindrod & Martin 2021) therefore appears to add to the redundancy of a set of resistance traits enabling them to survive under changing conditions.

How does recapping interfere with mite reproduction?

Overall, REC was suspected to increase MNR on several possible pathways. It could interfere with different parts of the complex reproductive phase of mites (summarised in Subchapter 1.1.1) by changing the kairomone levels (Le Conte et al. 2020) or by slightly altering the humidity and temperature inside brood cells. The opening of the cell cap could also lead mother mites to escape from the brood cell as has been supposed before (Guichard et al. 2022). However, this would result in orphan mite families or at least mite faeces inside recapped brood cells, which was hardly ever observed in the present studies. *Vice versa*, mites were also suspected to invade unsuitable brood ages when cells are opened and then getting trapped inside after recapping. However, this would have led to increased shares of infertile mites due to mismatches of host brood signals crucial for mite oogenesis (Sprau et al. 2021; Frey et al. 2013), which was also disproved in the present investigation (Chapter 3).
In fact, the suppressing effect of REC on mite reproduction was only apparent in brood cells

with delayed reproduction or missing males (Chapter 3, Fig. 1c, d), while no such effect was detectable in infertile cells (Chapter 3, Fig. 1b). Thus, recapping mainly affects fertile mother mites (i.e., mites with offspring) and lowers their success in raising mated daughters (Fig. 5).

REC could thus either cause *Varroa* to start egg-laying with a substantial delay, sometimes also skipping the first egg (i.e., the male), or distort the offspring constellation of normally reproducing mother mites.
As the picture-based analysis of the timing of REC presented in Chapter 3 revealed, REC indeed mainly occurs after the first eggs (i.e., the male and the eldest daughter) are already laid (Chapter 3, Fig. 3a). Thus, rather than interfering with the oviposition of mother mites, REC affects the *Varroa* offspring. The significantly increased levels of missing males and delayed reproduction thus must be caused by a loss of the male or the eldest daughter, respectively.

As described in detail by Donzé & Guerin (1994), both eggs, later developing in the male and the first daughter mite, are deposited by *Varroa* mothers in the anterior cell section near the cap. This is seen as a form of parental care, to protect the first offspring from movements of the host larva spinning its cocoon (Donzé & Guerin 1994). Ironically, this part of the cell is however especially exposed to worker bees opening the cell cap during REC. Thus, eggs could easily be removed by adult bees performing this behaviour, as recently shown for experimentally inserted items (Sprau et al. 2023). Also, protonymphs hatching from these eggs are greatly challenged by crossing the legs of their host pupae to reach the communal feeding side in the posterior part of the brood cell (Donzé & Guerin 1994). Thus, they frantically move around in the anterior part (Donzé & Guerin 1994), which in case of REC again might easily lead to their being removed by worker bees or even crawling out of the brood cell on their own. Notably, such a loss of the first daughter or the male would mostly be sufficient to prevent successful reproduction on the cell level, since the remaining daughters were either too young to reach maturity in time (delayed reproduction) or adult daughters were missing a male for mating (no male), thus exactly matching the observations presented in Chapter 3.

In line with the mode of action behind REC, apparently affecting mainly the first *Varroa* descendants, bees also appear to target cells containing mite offspring with this behaviour. The investigation of REC in infested cells with failed reproduction (Chapter 3) strongly suggests such a targeted behaviour, since it proves higher respective REC values in case of delayed reproduction (47.54 %) or missing males (53.52 %) compared to infertile cells (40.13 %).

Recapping as a behavioural proxy for *Varroa*-sensitive hygiene

Notably, the direct effect of REC on MNR shown in Chapter 3 does not necessarily contradict the hypothesis that increased REC might constitute a proxy for increased VSH (Sprau et al. 2023; Martin et al. 2020).

In fact, the findings of Chapter 2 even suggest that the increase of MNR found during brood interruptions was rather caused by increased levels of VSH targeting reproductive mites than by REC alone, as discussed above (Subchapter 5.2). The expression of REC was significantly increased when the need for brood care suddenly dropped due to the brood interruptions. Hence, this spontaneous behavioural adaptation strongly resembles the work force allocation reported for VSH (Tison et al. 2022), but was not the primary cause of MNR. REC thus seems to lower directly the reproductive success of mites in some cases, yet this effect could easily be replaced or overshadowed in case of subsequent VSH which removes the respective cells.

Results of the picture-based brood analysis presented in Chapter 3 additionally support this hypothesis, since the general temporal pattern of brood termination resembled the timing of REC after cell capping (Chapter 3, Fig. 3b; except hatching bees at day 10 post capping, see Chapter 3 for a detailed discussion). Although VSH was not directly accounted for in this study, it positively correlated with cell termination rates reported in earlier studies (Kirrane et al. 2014). Given the temporal overlap of the expression of both behaviours (Chapter 3, Fig. 3) the termination of initially uncapped and infested cells (i.e., VSH) may thus in some cases be a second step following REC, as discussed above and supposed before (Sprau et al. 2023; Martin et al. 2020).

The presented results therefore prove a direct effect of REC on *Varroa*-reproduction by mainly targeting the mite offspring. Nevertheless, judging from the literature, they also point towards a comparatively stronger effect of VSH in some cases, making the expression of REC redundant for the colony. However, in the latter case, REC activity still seems to overlap with VSH expression and thus might be used as a valuable selection proxy in any case.

5.4 The potential of MNR and REC for targeted selection and sustainable beekeeping

As discussed above, MNR and REC appear to be ideal candidate traits for targeted selection, since they proved efficient in several naturally surviving populations (Grindrod & Martin 2021; Mondet et al. 2020a) and are comparatively easy to quantify (Mondet et al. 2020b; Büchler et al. 2017).
However, up to now, no substantial selection progress was reported for managed populations, although both traits are accounted for in several breeding programs (Le Conte et al. 2020), keeping the suitability of these traits for targeted selection still under debate (Guichard et al. 2022; Eynard et al. 2020).

Perspectives and limitations

The results presented in Chapter 2 and Chapter 3 clearly underline the beneficial effects of MNR and REC in managed colonies, already frequently described for surviving populations (Luis et al. 2022; Grindrod & Martin 2021) and recently also found to some extend in an extensive field study on breeding stocks selected for *Varroa*-resistance under real-world beekeeping conditions (Büchler 2022). However, in the latter case no significant differences in the expression of MNR were found between breeding lines which had already been selected for this trait and those which had not undergone such a selection (Büchler 2022). This again fits the results presented here (Chapters 2 & 3), as well as previous reports on the comparatively low repeatability of this resistance trait (Büchler et al. 2020a; Eynard et al. 2020), which points towards strong external effects rather than a genetic predisposition as the main factor for the expression of MNR. The latter, however, would be required at least to some extent for successful targeted selection (Büchler et al. 2010). In fact, a sufficiently high heritability of traits is one of the main prerequisites of targeted selection and breeding (Eynard et al. 2020; Hoppe et al. 2020) and thus adds to their biological relevance (Büchler et al. 2010), their natural variability (Rinderer 1986), the applicability of testing (Büchler et al. 2010) and controlled mating within the population (Plate et al. 2019).

Heritability vs. Testing effort

The notion of heritability describes the share of variance in the expression of a trait observed in a given population which is caused by the genetic variance (e.g., the degree of relation

between relatives) and typically ranges between 1 and 0 (Kräußlich 1994, Rinderer 1986). High heritability values reflect a strong genotype-dependent effect on the phenotypic expression of the trait, which is conducive to targeted selection, while low values point to strong environmental effects hindering such selection efforts. In addition, a knowledge of heritability holds great importance for the estimation of breeding values reflecting the latest state of the art method for successful targeted selection in resistance breeding (Hoppe et al. 2020).
Heritability analyses have been conducted for several resistance traits (summarised in [Mondet et al. 2020a]), yet they often revealed values too low compared to the required measurement effort. Thus, several traits not fitting the needs of targeted selection were discarded over the years, although they proved to be beneficial for colony health in surviving populations (Le Conte et al. 2020; Büchler et al. 2010). As discussed in more detail in Chapter 4, when evaluating the suitability of traits for targeted selection, the degree of heritability (i.e., the expected breeding progress) has to be rated in the light of testing effort (i.e., the labour to be invested). One example for this cost-benefit analysis is the selection for increased grooming behaviour (see Subchapter 1.2.4), which has been shown to be selectable (Morfin et al. 2020; Büchler 2000) and beneficial for surviving populations (Locke 2016). However, breeding efforts have mostly stopped since the achievable outcome is offset by tedious testing protocols (Morfin et al. 2020; Büchler et al. 2010; Aumeier 2001) and comparatively low heritability (Pritchard 2016; Ehrhardt et al. 2007).
On the other hand, the development of *Varroa*-infestation on the colony level, despite its low heritability (h^2= 0.11, [Hoppe et al. 2020]), has been under targeted selection for decades. Although the heritability is even lower than in case of grooming behaviour (h^2= 0.16, [Pritchard 2016]) this drawback for selection is balanced by simple testing procedures, enabling bigger data sets and thus nevertheless leading to substantial selection progress (Hoppe et al. 2020).

In the case of MNR and REC, the measurement of raw values is undisputedly more complex than are simple infestation measurements needed to trace the development of *Varroa*-infestation (Mondet et al. 2020b; Büchler et al. 2017; Dietemann et al. 2013). Nevertheless, both traits can be accounted for based on frozen brood combs, enabling investigations temporally independent from the sampling date (Büchler et al. 2017). Especially for mass sampling during high beekeeping season, this holds great importance for the sample volume, which is processable in scientific investigations as well as breeding programs.
In contrast to measurements of VSH (Sprau et al. 2021; Dietemann et al. 2013; Villa et al. 2009) the sampling for MNR and REC (Mondet et al. 2020b; Büchler et al. 2017) is also easier since no additional manipulation of brood cells, e.g., artificial infestation, is needed in advance and samples can be taken in one working step.
Thus, for application in practice, the sampling requirements of MNR and REC are comparably easy to implement in large-scale selection approaches. However, the heritability of both traits remained uncertain for long, although MNR and REC hold all the above-mentioned properties favourable for selection and are currently already focussed by laborious breeding efforts (Le Conte et al. 2020; Mondet et al. 2020a). While the heritability of MNR has so far only once been investigated, based on a comparatively small dataset of 28 queens (h^2= 0.46, [(Harbo and Harris 1999]) no investigations of REC in infested brood cells (RECinf, see Tab. 1) have been reported. However, Guichard et al. (2021) evaluated the heritability of REC in all cells (RECall, see Tab. 1) and reported it to be quite low ($h^2 \approx 0.05$). Yet, the overall frequency of REC in this study population of 121 colonies was distinctively lower than the average values described for *Varroa*-susceptible populations worldwide (<10 % [Guichard et al. 2021] compared to 33 %

[Grindrod & Martin 2021]). In addition, RECinf seems to be of greater interest for selection, since this targeted behaviour is more important for a decreased reproduction of mites (Oddie et al. 2021) and appears to be less affected by colony level infestation, as shown in Chapter 4 of the present work (Chapter 4, Tab. 3).

Heritability of mite non-reproduction and recapping

Chapter 4 represents the first investigation of the respective heritability of MNR and REC based on an extensive dataset. Brood investigation data from more than 4,400 Carnica and Buckfast colonies was compiled with their respective pedigree information to calculate these values. Due to differences in their genetic background and respective breeding histories, both populations differed in the heritability of each trait. Thus, the heritability accessible for selection was found to be 0.44 and 0.18 for MNR, as well as 0.40 and 0.44 for RECinf in Carnica and Buckfast stock, respectively (Chapter 4, Tab. 2, 4). At a first glance, the traits therefore appeared to be surprisingly heritable given the outer effects altering phenotypic values shown in Chapters 2 & 3. In addition, the highest heritability values known for other beekeeping traits in an extensively studied Carnica population are displayed by 0.28 for calmness on the combs and gentleness, respectively (Hoppe et al. 2020). Thus, the values presented in Chapter 4 appear to overestimate the respective heritability values, since the data structure in this study, in contrast to the long-term investigations by (Hoppe et al. 2020), did not allow for the consideration of apiary and examinator effects. As discussed in detail in Chapter 4, the heritability values are therefore likely to be inflated as a result of neglecting such effects in the calculations. However, this overestimation can be assessed by comparing the heritability for hygienic behaviours of bees towards pin-killed brood (AGT 2020), which was calculated for a subset of colonies in both studies (Chapter 4; [Hoppe et al. 2020]). As presented in Chapter 4, this trait was found to be highly heritable in the present study ($h^2 = 0.72$), yet based on the values described earlier ($h^2 = 0.21$ [Hoppe et al. 2020]), it appears to be overestimated by approximately 3.4 times. By adjusting the most likely inflated heritability values for MNR and REC by this factor, the values appear considerably smaller but more realistic and congruent with the findings presented in Chapters 2 & 3. Except for the heritability of MNR in the Buckfast population, all MNR and REC values adjusted like this remain, however, in the range of the heritability of honey yield described earlier (Hoppe et al. 2020). With $h^2= 0.14$, this heritability reflects apparently strong outer effects like changing nectar resources and weather conditions which alter the honey yield regardless of the colony's genetic background (Hoppe et al. 2020). On the other hand, honey yield is one of the most accounted for traits in targeted breeding and proved to be selectable after substantial genetic progress was made in the last decades. As nicely demonstrated by Hoppe et al. (2020), the genetic effect of these breeding efforts caused an increase of over 5 kg in the yearly averages of honey yield when comparing the breeding population in 2018 to the state of 1992. This impressive progress was initiated in the early 1990s, when breeders started to select based on breeding values instead of raw values, as had been common before (Hoppe et al. 2020). Especially for traits with a low heritability, such breeding values paint a clearer picture of the genotypic potential of colonies than raw values of the individual colony alone, since they additionally incorporate test information from relatives and unrelated colonies tested in parallel (Bienefeld et al. 2007). Thus, by testing related colonies under different environmental conditions, outer effects altering the trait expression, such as shown in Chapters 2 & 3 for REC and MNR, can be considered to adjust the breeding values accordingly.

Implications for applied breeding

The results presented in Chapter 4 revealed that both MNR and REC hold sufficiently high heritability for targeted selection. However, as shown by (Hoppe et al. 2020), the selection decisions should rather be based on breeding values than on raw values, since both MNR and REC are strongly affected by outer factors as shown in Chapters 2 & 3.

In fact, as shown in Fig. 3 and Fig. 5 of Chapter 4, the retrospective estimation of breeding values for MNR and REC reveals a positive genetic trend over the past years, although this is hardly detectable in the raw values of both traits. This again underlines the risks proceeding from misleading raw values as well as the potential for targeted selection based on breeding values. The predictivity of breeding values, however, relies on the extent and quality of raw data deriving from comparable performance test methods (e.g., AGT 2020).
Both the systematic continuation of MNR and REC measurements and the implementation of apiary and examinator effects are thus highly recommended for future breeding efforts, since this may substantially improve the predictivity of breeding values as well as the selection progress for these traits. However, even the comparatively low predictivities found for MNR and REC breeding values in Chapter 4 are still comparable to those of *Varroa*-infestation development, a trait successfully selected based on the corresponding breeding values for long (Hoppe et al. 2020). In addition to the results gained from retrospective breeding value estimations discussed above, this strongly suggests that breeding values for MNR and REC, even though displaying a low predictivity compared to other traits, are still more reliable for selection decisions than the respective raw values.

Implications for applied beekeeping

Besides their relevance for resistance breeding as discussed above, MNR and REC may also be fostered in hive management. For example, the results presented in Chapter 2 clearly display the beneficial effects of brood interruptions for the colony. By immediately increasing the frequency of REC and suppressing mite reproductive success in the long term, such brood interruptions proved to decrease the infestation level of colonies significantly (Chapter 2, Fig. 5). Although usually combined with oxalic acid applications, induced brood interruptions are well known summer treatments for *Varroa*-control (Büchler et al. 2020b). In the light of the results presented here (Chapter 2), brood interruptions might even be sufficiently effective without subsequent drug applications in case of low infestation levels. To induce such brood interruptions, queens are commonly caged two weeks before the last honey harvest of the season to prevent negative effects on the honey yield (Kovačić et al. 2023). In most parts of Germany, for example, this last honey flow ends with the withering of the lime tree (*Tilia cordata*) blossom in mid-July. Thus, following the results presented in Chapter 2, a brood interruption induced at the beginning of July might hold the potential to increase MNR values for approximately 30 days and so could cover a phase in which MNR might otherwise often be expressed at comparatively low levels (Chapter 3, Fig. 1a; [Otten 1991]). However, as described above, the occurrence of REC and MNR appears to be altered by several factors and *Varroa*-infestation levels can fluctuate rapidly in this time of the season (Frey & Rosenkranz 2014). Although brood interruptions proved to be beneficial in terms of lower infestation levels and increased reproductive failure of mites, further research is thus needed to examine the potential and limits of treatment-free hive management techniques aiming for higher MNR and REC values.

MNR and REC are thus valuable resistance traits which can be used for targeted selection and may be fostered by biotechnical hive management, to jointly achieve a more sustainable beekeeping with lowered drug application. In doing so, however, the limitations of the comparatively low heritabilities of both traits have to be considered, and selection decisions should mainly be based on breeding values deriving from robust performance testing data.

5.5 Conclusion and Outlook

My doctoral thesis sheds light on the complex mechanisms of *Varroa*-resistance by focussing on two of the most prominent traits in this field: decreased reproductive success of mites (mite non-reproduction; MNR) and the recapping of sealed brood cells (REC). Knowledge gaps concerning outer effects which might alter the expression of MNR and REC, as well as possible interactions between both traits, were filled by extensive field studies. Beginning with manipulations of host brood availability as a prerequisite for mite reproduction, Chapter 2 presents clear evidence for an increasing expression of REC and MNR due to brood interruptions. In this, the interruption of brood rearing in host colonies proved to increase the expression of MNR both immediately and in the long-term, which was attributed to a significant increase of mite infertility. In contrast, neither the occurrence of cells with delayed-developing daughters, nor of cells without male offspring was affected by brood interruptions. In addition, the significantly increased expression of REC as a short-term reaction to brood interruptions showed no apparent effect on any form of MNR. However, based on earlier studies fitting well to these observations, it appears reasonable to assume that *Varroa*-sensitive hygiene (VSH), as one of the main effects overlapping with REC, might have overshadowed direct effects of REC in this case. Although not accounted for in the present work, previous studies have suggested VSH as a main cause for MNR (Harbo & Harris 2005), which preferably targets successfully reproducing mites (i.e., selects out remaining infertile mites [Mondet et al. 2020b; Oddie et al. 2018]) and depends on work force allocation (Tison et al. 2022). Thus, future studies on factors eliciting VSH and the expression of this trait under changing brood amounts would further elucidate the puzzle of resistance traits displayed under changing conditions. In this context, the long-term studies of MNR and REC expression presented in Chapter 3 were aimed at shedding light on possible changes. Indeed, they have highlighted such temporal changes by revealing strong seasonal variation in both traits. Despite the overall variability, this long-lasting investigation covering a wide range of real-world situations (e.g., differing nectar flows and brood amounts) also clearly proved the importance of REC as a stand-alone resistance trait which permanently increases MNR occurrence. Importantly, however, REC was only found to increase the shares of delayed-developing daughter mites and missing males, rather than affecting infertile mites. Thus, the findings of Chapters 2 & 3 revealed different external factors which alter the underlying causes of MNR differently, pointing towards a redundancy system of complementary *Varroa*-resistance mechanisms (Fig. 5). In this context, I recommend to evaluate and account for the particular causes of MNR (i.e., infertility, delayed reproduction or missing males) separately in future studies, since their respective occurrence appears to hold additional information concerning the underlying mechanisms.

In line with these findings, the results gained from the novel picture-based investigation of REC (Chapter 3) showed that uncapping of brood cells mainly occurred when mite offspring would be expected in such cells. Although these results generally point to the two eldest mite descendants as the primary targets of REC, the exact mode of action behind this trait still remains subject to speculation. Thus, further studies on the why and how of REC and its effects on mites are needed to uncover the triggers inducing this behaviour and the fate of mite

offspring in such cells. For example, these questions could be addressed by artificial uncapping of infested brood cells to investigate the reaction of mite offspring (e.g., emigration from brood cells) or artificial transfer of mite offspring to uninfested brood cells to investigate the reaction of bees (e.g., opening cells to remove mite offspring).
While the results of Chapters 2 & 3 identified factors which alter the expression of MNR and REC, thereby clearly proving them to be linked, these findings also suggest a strong outer influence on the expression of both traits, hindering their targeted selection in practice. Consequently, in Chapter 4 the heritability of both traits was investigated based on a large sample set to weigh their usability in breeding against the limits revealed in the previous Chapters (2 & 3).
In this, REC and MNR proved for the first time to be heritable and thus selectable traits which are frequently expressed in German Buckfast and Carnica populations. However, as suggested by the findings of Chapters 2 & 3, the respective heritabilities appear to be quite low, yet comparable to the well-established breeding parameter honey yield. Thus, I highly recommend to adjust selection decisions for outer effects altering the phenotype of traits as far as possible, in order to achieve any considerable breeding progress. As discussed in Chapter 4, breeding value estimation presents a powerful tool for such adjustments of MNR and REC raw values (Chapter 4, Fig. 3, 5). However, such calculations rely on solid data bases deriving from a proper acquisition of phenotypic values in order to increase the predictivity of breeding values. Thus, standardised methods (e.g., AGT 2020; Büchler et al. 2017) which implement the findings of this work should be used to gather more comprehensive data sets and improve breeding-value-based selection decisions. In this context, the findings of Chapter 2 concerning the increasing effect of brood interruptions on MNR play a dual role, since they can be implemented in hive management to decrease the reproductive success of mites, yet need to be considered when measuring MNR in breeding colonies (Fig. 5). In the latter case, a recovery phase for the mite population after artificial infestations as well as shortened brood interruption in mite-donor colonies might substantially contribute to more detailed data acquisition.

In conclusion, these findings greatly extend our understanding of *Varroa*-resistance mechanisms in honeybees. In addition to important implications for *Varroa*-research, the present work has opened up new avenues for targeted breeding towards MNR and REC, as well as for the use of these traits in sustainable beekeeping.

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Appendix

List of abbreviations, for detailed definitions see Tab. 1.

Notation	Description	Page List
MNR	**m**ite **n**on-**r**eproduction	V, 7-13, 16&17, 28&29, 31, 34&35, 37, 42, 46-52, 56, 100, 110-125
SMR	**s**uppressed **m**ite **r**eproduction	7&8, 10, 18, 21, 39, 41, 46, 54, 99-107, 109, 126, 129
REC	**rec**apping	V, 11-17, 28, 32, 34, 36&37, 46&47, 49-52, 88, 100&101, 106&107, 110-114, 116-125
RECinf	**rec**apping of **inf**ested cells	11, 15, 49&50, 53, 99-101, 103&104, 106, 121&122
RECall	**rec**apping of **all** cells	11, 15, 49, 53, 99-101, 104-106, 121
VSH	*V**arroa*-**s**ensitive **h**ygiene	11-14, 22&23, 34, 36, 41, 47, 51, 54, 100, 108, 114-117, 119-121, 124, 130

Affidavit

I hereby declare that my thesis entitled: „Behavioural resistance to *Varroa destructor* in the Western honeybee *Apis mellifera* - Mechanisms leading to decreased mite reproduction" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore I verify that the thesis has not been submitted as part of another examination process neither in identical nor in similar form.

Besides I declare that if I do not hold the copyright for figures and paragraphs, I obtained it from the rights holder and that paragraphs and figures have been marked according to law or for figures taken from the internet the hyperlink has been added accordingly.

_____ _____

Place, Date Signature PhD-student

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation: „Resistenzverhalten der Westlichen Honigbiene *Apis mellifera* gegen *Varroa destructor* - Zu verringerter Milbenreproduktion führende Mechanismen ", eigenständig, d. h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen, als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

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Ort, Datum Unterschrift Doktorand

Curriculum Vitae

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Gabel, M.; Scheiner, R. and Büchler, R. (2023): **Immediate and long-term effects of induced brood interruptions on the reproductive success of *Varroa destructor*.** Apidologie, 54(2):20
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Gabel, M. & Hoppe, A.; Scheiner, R.; Obergfell, J. and Büchler, R. (2023): **Heritability of *Apis mellifera* recapping behavior and suppressed mite reproduction as resistance traits towards *Varroa destructor*.** Frontiers in Insect Science, 3:1135187.
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This article can be downloaded here: https://doi.org/10.3390/microorganisms11112780

Gabel, M.; Scheiner, R.; Steffan-Dewenter, I. and Büchler, R. (2023): **Reproduction of Varroa destructor depends on well-timed host cell recapping and seasonal patterns**. Scientific reports, 13(2023):22484
This article can be downloaded here: https://doi.org/10.1038/s41598-023-49688-9

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*Büchler, R.; Andonov, S.; Bernstein, R.; Bienefeld, K.; Costa, C.; Du, M.; **Gabel, M.**; Given, K.; Hatjina, F.; Harpur, B.; Hoppe, A.; Kezic, N.; Kovacic, M.; Kryger, P.; Mondet, F.; Spivak, M.; Uzunov, A.; Wegener, J. and Wilde, J. (accepted):
Standard methods for rearing and selection of *Apis mellifera* queens.
Journal of Apicultural Research, Manuscript: TJAR-2022-0143

*These publications are not part of this thesis.

Descriptions of the Specific Contributions of the PhD-Candidate to a Publication with Several Co-Authors and Confirmation by the Co-Authors

Description of the specific contributions of the PhD-candidate to a publication with several co-authors and confirmation by the co-authors

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Title of the publication:

Immediate and long-term effects of induced brood interruptions on the reproductive success of *Varroa destructor*

Names of Co-Authors:

Ricarda Scheiner, Ralph Büchler

Publication details	Description of the own contribution
Writing of the article Which parts of the article have been written to which extent by the candidate?	Martin Gabel prepared the original draft and performed reviews and editing.
Performed research Which experimental procedures have been conducted by the candidate?	Martin Gabel prepared and performed the fieldwork, including treatment of colonies and sampling as well as data management.
Conceptual design of the research To which extent did the candidate contribute to the conceptional design of the research project?	Martin Gabel and Ralph Büchler performed conceptualization to equal shares.
Data analysis To which extent did the candidate contribute to the data analysis?	Martin Gabel compiled raw data and performed statistical analyses and visualization.
Overall contribution of the candidate (in%)	80 %

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Herewith I confirm that the above description of the specific contributions of the PhD-candidate to the publication is correct,

Name of responsible author Signature Date

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Title of the publication:

Reproduction of *Varroa destructor* depends on well-timed host cell recapping and seasonal patterns

Names of Co-Authors:

Ricarda Scheiner, Ingolf Steffan-Dewenter, Ralph Büchler

Publication details	**Description of the own contribution**
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Performed research Which experimental procedures have been conducted by the candidate?	Martin Gabel planned and performed the fieldwork, including hive management and sampling as well as data management and picture analysis.
Conceptual design of the research To which extent did the candidate contribute to the conceptional design of the research project?	Martin Gabel and Ralph Büchler performed conceptualization to equal shares.
Data analysis To which extent did the candidate contribute to the data analysis?	Martin Gabel compiled raw data and performed statistical analyses and visualization.
Overall contribution of the candidate (in%)	80 %

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Description of the specific contributions of the PhD-candidate to a publication with several co-authors and confirmation by the co-authors

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Heritability of *Apis mellifera* recapping behavior and suppressed mite reproduction as resistance traits towards *Varroa destructor*

Names of Co-Authors:

Andreas Hoppe, Ricarda Scheiner, Jörg Obergfell, Ralph Büchler

Publication details	**Description of the own contribution**
Writing of the article Which parts of the article have been written to which extent by the candidate?	Martin Gabel prepared the original draft and performed reviews and editing. Details on the methods used for breeding value estimation and calculation of heritability were authored by Andreas Hoppe.
Performed research Which experimental procedures have been conducted by the candidate?	Martin Gabel prepared common protocols and databases for raw data acquisition, collected field data and supported breeding groups in the context of the SMR-Project. He compiled these raw data and pedigree information for the Carnica population, as well as together with Jörg Obergfell for the Buckfast population.
Conceptual design of the research To which extent did the candidate contribute to the conceptional design of the research project?	Martin Gabel and Ralph Büchler performed conceptualization to equal shares.
Data analysis To which extent did the candidate contribute to the data analysis?	Martin Gabel structured, reviewed and compiled raw data for following heritability calculations performed by Andreas Hoppe.
Overall contribution of the candidate (in%)	50 %

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Ralph Büchler		

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Herewith I confirm that the above description of the specific contributions of the PhD-candidate to the publication is correct,

Name of responsible author Signature Date

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